

"Express Mail" Mailing Label No. **EL 873 906 720 US**
Date of Deposit **August 28, 2001**

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Susan LaMont
Typed or Printed Name of Person Mailing Paper or Fee

Susan LaMont
Signature of Person Mailing Paper or Fee

Application for U.S. Letters Patent Entitled

GENE IDENTIFICATION

claiming priority to U.S. Patent Application S.N. 09/395,448 filed September 14, 1999

by Inventors:

Casey C. Case
Fyodor Urnov

ROBINS & PASTERNAK LLP
90 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: 650-325-7812
Facsimile: 650-325-7823

Attorney Docket No. S7-US3; 8325-0007.20

GENE IDENTIFICATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of US patent application Serial No.

5 09/395,448, filed September 14, 1999, the disclosure of which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

The present disclosure is in the field of functional genomics and gene identification.

BACKGROUND

Determining the function of a gene of interest is important for identifying potential genomic targets for drug discovery. Genes associated with a particular function or phenotype can then be validated as targets for discovery of therapeutic compounds. Historically, the function of a particular gene has been identified by associating expression of the gene with a specification function of phenotype in a biological system such as a cell or a transgenic animal.

One known method used to validate the function of a gene is to genetically
20 remove the gene from a cell or animal (i.e., create a “knockout”) and determine whether
or not a phenotype (i.e., any change, e.g., morphological, functional, etc., observable by
an assay) of the cell or animal has changed. This determination depends on whether the
cell or organism survives without the gene and is not feasible if the gene is required for
survival. Other genes are subject to counteracting mechanisms that are able to adapt to
25 the disappearance of the gene and compensate for its function in other ways. This
compensation may be so effective, in fact, that the true function of the deleted gene may
go unnoticed. The technical process of creating a “knockout” is laborious and requires
extensive sequence information, thus commanding immense monetary and technical
resources if undertaken on a genome wide scale.

In another example, antisense methods of gene regulation and methods that rely on targeted ribozymes are highly unpredictable. Another method for experimentally determining the function of a newly discovered gene is to clone its cDNA into an expression vector driven by a strong promoter and measure the physiological consequence of its over-expression in a transfected cell. This method is also labor intensive and does not address the physiological consequences of down-regulation of a target gene. Therefore, simple methods allowing the selective over- and under-expression of uncharacterized genes would be of great utility to the scientific community. Methods that permit the regulation of genes in cell model systems, transgenic animals and 5 transgenic plants would find widespread use in academic laboratories, pharmaceutical companies, genomics companies and in the biotechnology industry.

10

An additional use of target validation is in the production of *in vivo* and *in vitro* assays for drug discovery. Once the gene causing a selected phenotype has been identified, cell lines, transgenic animals and transgenic plants could be engineered to 15 express a useful protein product or repress a harmful one. These model systems are then used, e.g., with high throughput screening methodology, to identify lead therapeutic compounds that regulate expression of the gene of choice, thereby providing a desired phenotype, e.g., treatment of disease.

Methods currently exist in the art, which allow one to alter the expression 20 of a given gene, e.g., using ribozymes, antisense technology, small molecule regulators, over-expression of cDNA clones, and gene-knockouts. As described above, these methods have to date proven to be generally insufficient for many applications and typically have not demonstrated either high target efficacy or high specificity *in vivo*. For useful experimental results and therapeutic treatments, these characteristics are desired.

25 Gene expression is normally controlled by sequence specific DNA binding proteins called transcription factors. These bind in the general proximity (although occasionally at great distances) of the point of transcription initiation of a gene and typically include both a DNA binding domain and a regulatory domain. They act to influence the efficiency of formation or function of a transcription initiation complex at 30 the promoter. Transcription factors can act in a positive fashion (transactivation) or in a negative fashion (transrepression). Although transcription factors typically contain a

regulatory domain, repression can also be achieved by steric hindrance via a DNA binding domain alone.

Transcription factor function can be constitutive (always “on”) or conditional. Conditional function can be imparted on a transcription factor by a variety of means, but the majority of these regulatory mechanisms depend of the sequestering of the factor in the cytoplasm and the inducible release and subsequent nuclear translocation, DNA binding and transactivation (or repression). Examples of transcription factors that function this way include progesterone receptors, sterol response element binding proteins (SREBPs) and NF- κ B. There are examples of transcription factors that respond to phosphorylation or small molecule ligands by altering their ability to bind their cognate DNA recognition sequence (Hou *et al.*, *Science* 256:1701 (1994); Gossen & Bujard, *Proc. Natl. Acad. Sci. U.S.A.* 89:5547 (1992); Oligino *et al.*, *Gene Ther.* 5:491-496 (1998); Wang *et al.*, *Gene Ther.* 4:432-441 (1997); Neering *et al.*, *Blood* 88:1147-1155 (1996); and Rendahl *et al.*, *Nat. Biotechnol.* 16:757-761 (1998)).

Zinc finger proteins (“ZFPs”) are proteins that can bind to DNA in a sequence-specific manner. Zinc fingers were first identified in the transcription factor TFIIIA from the oocytes of the African clawed toad, *Xenopus laevis*. Zinc finger proteins are widespread in eukaryotic cells. An exemplary motif characterizing one class of these proteins (Cys₂His₂ class) is -Cys-(X)₂₋₄-Cys-(X)₁₂-His-(X)₃₋₅-His (SEQ ID NO: 1) (where X is any amino acid). A single finger domain is about 30 amino acids in length and several structural studies have demonstrated that it contains an alpha helix containing the two invariant histidine residues co-ordinated through zinc with the two cysteines of a single beta turn. To date, over 10,000 zinc finger sequences have been identified in several thousand known or putative transcription factors. Zinc finger proteins are involved not only in DNA-recognition, but also in RNA binding and protein-protein binding. Current estimates are that this class of molecules will constitute the products of about 2% of all human genes.

The X-ray crystal structure of Zif268, a three-finger domain from a murine transcription factor, has been solved in complex with its cognate DNA-sequence and shows that each finger can be superimposed on the next by a periodic rotation and translation of the finger along the main DNA axis. The structure suggests that each finger

interacts independently with DNA over 3 base-pair intervals, with side-chains at positions -1, 2, 3 and 6 on each recognition helix making contacts with respective DNA triplet sub-site. The amino terminus of Zif268 is situated at the 3' end of its DNA recognition subsite. Recent results have indicated that some zinc fingers can bind to a fourth base in a 5 target segment (Isalan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:5617-5621 (1997). The fourth base is on the opposite strand from the other three bases recognized by zinc finger and complementary to the base immediately 3' of the three base subsite.

The structure of the Zif268-DNA complex also suggested that the DNA sequence specificity of a zinc finger protein might be altered by making amino acid 10 substitutions at the four helix positions (-1, 2, 3 and 6) on a zinc finger recognition helix. Phage display experiments using zinc finger combinatorial libraries to test this observation were published in a series of papers in 1994 (Rebar *et al.*, *Science* 263:671-673 (1994); Jamieson *et al.*, *Biochemistry* 33:5689-5695 (1994); Choo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 91:11163-11167 (1994)). Combinatorial libraries were constructed with 15 randomized side-chains in either the first or middle finger of Zif268 and then isolated with an altered Zif268 binding site in which the appropriate DNA sub-site was replaced by an altered DNA triplet. Correlation between the nature of introduced mutations and the resulting alteration in binding specificity gave rise to a partial set of substitution rules for rational design of zinc finger proteins with altered binding specificity. Greisman & 20 Pabo, *Science* 275:657-661 (1997) discuss an elaboration of a phage display method in which each finger of a zinc finger protein is successively subjected to randomization and selection. This paper reported selection of zinc finger proteins for a nuclear hormone response element, a p53 target site and a TATA box sequence.

Recombinant zinc finger proteins have been reported to have the ability to 25 regulate gene expression of transiently expressed reporter genes in cultured cells (see, e.g., Pomerantz *et al.*, *Science* 267:93-96 (1995); Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530 1997); and Beerli *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633 (1998)). For example, Pomerantz *et al.*, *Science* 267:93-96 (1995) report an attempt to 30 design a novel DNA binding protein by fusing two fingers from Zif268 with a homeodomain from Oct-1. The hybrid protein was then fused with either a transcriptional activator or repressor domain for expression as a chimeric protein. The

TOP SECRET - DRAFT

chimeric protein was reported to bind a target site representing a hybrid of the subsites of its two components. The authors then constructed a reporter vector containing a luciferase gene operably linked to a promoter and a hybrid site for the chimeric DNA binding protein in proximity to the promoter. The authors reported that their chimeric
5 DNA binding protein could activate or repress expression of the luciferase gene.

Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530 (1997) report forming a composite zinc finger protein by using a peptide spacer to link two component zinc finger proteins, each having three fingers. The composite protein was then further linked to transcriptional activation or repression domains. It was reported that the
10 resulting chimeric protein bound to a target site formed from the target segments bound by the two component zinc finger proteins. It was further reported that the chimeric zinc finger protein could activate or repress transcription of a reporter gene when its target site was inserted into a reporter plasmid in proximity of a promoter operably linked to the reporter.

15 Beerli *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633 (1998) report construction of a chimeric six finger zinc finger protein fused to either a KRAB, ERD, or SID transcriptional repressor domain, or the VP16 or VP64 transcriptional activation domain. This chimeric zinc finger protein was designed to recognize an 18 bp target site in the 5' untranslated region of the human erbB-2 gene. Using this construct, the authors
20 of this study report both activation and repression of a transiently expressed reporter luciferase construct linked to the erbB-2 promoter.

In addition, a recombinant zinc finger protein was reported to repress expression of an integrated plasmid construct encoding a bcr-abl oncogene (Choo *et al.*, *Nature* 372:642-645 (1994)). The target segment to which the zinc finger proteins bound
25 was a nine base sequence GCA GAA GCC chosen to overlap the junction created by a specific oncogenic translocation fusing the genes encoding bcr and abl. The intention was that a zinc finger protein specific to this target site would bind to the oncogene without binding to abl or bcr component genes. The authors used phage display to select a variant zinc finger protein that bound to this target segment. The variant zinc finger
30 protein thus isolated was then reported to repress expression of a stably transfected bcr-abl construct in a cell line.

To date, these methods have focused on regulation of either transiently expressed, known genes, or on regulation of known exogenous genes that have been integrated into the genome. In contrast, specific regulation of a candidate gene or list of genes to identify the cause of a selected phenotype has not been demonstrated in the art.

5 Therefore, a need exists for useful methods of identifying the biological function of a selected gene or genes and or validating a gene or genes as a suitable target for drug discovery.

Furthermore, the determination of a draft nucleotide sequence of the human genome opens up the prospect of identifying all human genes. *See*, for example,

10 10 *Science* 291:1177-1351 (2001) and *Nature* 409:813-958 (2001). Identification of, for example, disease-related genes could lead to the discovery of new therapeutics. Some genes have already been identified based on protein and/or RNA expression; while others have been and can be identified by homology to other human genes or to related genes in other organisms.

15 15 However, many problems in unambiguously identifying human genes still exist and as a result, a complete list of human genes is not currently available, nor is it likely to become available in the near future. For example, the use of expressed sequence tag (EST) sequences to predict the existence of a gene is subject to artifacts arising from unspliced RNA, non-gene-derived transcription and contamination of cDNA preparations,

20 20 from which ESTs are derived, with genomic DNA. The use of sequence similarity to known genes as a criterion for identifying new genes rules out the possibility of identifying any new gene for which a homologous sequences is not already known.

Various gene prediction algorithms have been devised, but their success rate in identifying new genes is unacceptably low. Thus, currently-available methods for

25 25 predicting the existence of a gene, based on analysis of genome sequence, are not particularly effective. *See*, in particular, *Nature* 409 *supra* p. 819 (“When is a predicted gene a gene?”) and pp. 892-907 (“Gene content of the human genome”); Galas (2001) *Science* 291:1257-1260; and Goodman (2001) *Genome Technology* July 2001:52-55.

Accordingly, there is a need for methods to confirm putative gene assignments

30 30 that are based on gene prediction algorithms, sequence homology, ESTs and related techniques.

SUMMARY

In one aspect, described herein is a method for identifying a gene. In certain embodiments, the method comprises: (a) obtaining a putative gene sequence (PGS); (b) 5 contacting a cell with an exogenous molecule, wherein the cell comprises the putative gene sequence, and wherein the exogenous molecule binds to and modulates expression of the putative gene sequence; and (c) assaying the cell for at least one selected phenotype, wherein, if one or more of the selected phenotypes are observed, the putative gene sequence is identified as a gene. The putative gene sequence can be obtained, for 10 example, from a gene prediction algorithm; by analysis of expressed sequence tags; and/or by homology. In any of the methods described herein, the gene can encode, for example, a protein or an RNA (*e.g.*, structural RNA, regulatory RNA, enzymatic RNA, antisense RNA, ribozyme, ribosomal RNA or transfer RNA) and the cell can be, for example, an animal cell (*e.g.*, a mammalian cell such as a human cell), a plant cell, a 15 bacterial cell, a protozoal cell, or a fungal cell. The exogenous molecule can be, for example, a zinc finger protein.

In certain embodiments, the exogenous molecule binds near the putative transcription startsite of the PGS. In other embodiments, the exogenous molecule binds in the putative transcribed region of the PGS (*e.g.*, in the putative coding region of the 20 PGS). In still further embodiments, the exogenous molecule binds in a putative nontranscribed regulatory region of the PGS.

In further embodiments, the exogenous molecule comprises an activation domain (*e.g.*, VP16, p65 and functional fragments thereof); a repression domain (*e.g.*, KRAB, v-erbA and functional fragments thereof); or a bifunctional domain (BFD), such as thyroid 25 hormone receptor, retinoic acid receptor, estrogen receptor, glucocorticoid receptor and functional fragments thereof, in which the activity of the bifunctional domain is dependent upon interaction of the BFD with a second molecule (*e.g.*, a protein or a small molecule such as 3,5,3'-triodo-L-thyronine (T3), all-*trans*- retinoic acid, estradiol, tamoxifen, 4-hydroxy-tamoxifen, RU-486 or dexamethasone).

30 In further embodiments, the phenotype is a change in a property, for example, cell growth, cell cycle control, cellular physiology and cellular response to a pathogen. In

other embodiments, the phenotype is expression of a RNA molecule. In yet other embodiments, the phenotype is an alteration in the transcriptional program of the cell.

In still further embodiments, the cell is infected with a virus and the gene is a viral gene.

5 These and other embodiments will be readily apparent to one of skill in the art upon reading the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematic representation of target validation using zinc finger proteins to regulate gene expression.

10 Figure 2 shows zinc finger protein expression constructs.

Figure 3 shows luciferase reporter constructs for zinc finger protein regulation of gene expression.

15 Figure 4 shows the effect of zinc finger proteins on luciferase reporter gene activation.

Figure 5 shows activation of a human VEGF native reporter gene by zinc finger proteins.

DETAILED DESCRIPTION

20 **Introduction**

As described herein, the present disclosure provides zinc finger proteins used in assays to determine the phenotypic consequences and function of gene expression. The recent advances in analytical techniques, coupled with focused mass sequencing efforts have created the opportunity to identify and characterize many more molecular targets than were previously available. This new information about genes and their functions will speed along basic biological understanding and present many new targets for therapeutic intervention. In some cases analytical tools have not kept pace with the generation of new data. An example is provided by recent advances in the measurement of global differential gene expression. These methods, typified by gene expression microarrays, differential cDNA cloning frequencies, subtractive hybridization and differential display methods, can very rapidly identify genes that are up or down-

regulated in different tissues or in response to specific stimuli. Increasingly, such methods are being used to explore biological processes such as, transformation, tumor progression, the inflammatory response, neurological disorders etc. One can now very easily generate long lists of differentially expressed genes that correlate with a given 5 physiological phenomenon, but demonstrating a causative relationship between a differentially expressed gene and the phenomenon is difficult. Until now, simple methods for assigning function to differentially expressed genes have not kept pace with the ability to monitor differential gene expression.

However, zinc finger protein technology can be used to rapidly analyze 10 differential gene expression studies. Engineered zinc finger proteins can be readily used to up or down-regulate any candidate target gene. Very little sequence information is required to create a gene-specific DNA binding domain. This makes the zinc finger protein technology ideal for analysis of long lists of poorly characterized differentially expressed genes. One can simply build a zinc finger-based DNA binding domain for 15 each candidate gene, create chimeric up and down-regulating artificial transcription factors and test the consequence of up or down-regulation on the phenotype under study (transformation, response to a cytokine etc.) by switching the candidate genes on or off one at a time in a model system.

Additionally, greater experimental control can be imparted by zinc finger 20 proteins than can be achieved by more conventional methods. This is because the production and/or function of an engineered zinc finger protein can be placed under small molecule control. Examples of this approach are provided by the Tet-On system, the ecdysone-regulated system and a system incorporating a chimeric factor including a mutant progesterone receptor. These systems are all capable of indirectly imparting small 25 molecule control on any candidate gene of interest or any transgene by placing the function and/or expression of a zinc finger protein regulator under small molecule control. In one embodiment, a cell comprises two zinc finger proteins. The zinc finger proteins either target two different candidate genes (i.e., two genes associated with the same phenotype), or two different target sites on the same candidate gene. Each zinc 30 finger protein also comprises a regulatory domain. Expression of each zinc finger protein

RECEIVED - CASES
FEB 2002

is under different small molecule control, allowing variations in the degree of activation or repression of gene expression.

The present application therefore provides for the first time methods of using zinc finger proteins for identifying a gene or genes associated a selected phenotype, 5 e.g., for drug discovery target validation or for functional genomics. The present disclosure provides zinc finger DNA binding proteins that have been engineered to specifically recognize genes, with high efficacy. Modulation of gene expression using zinc finger proteins is used to determine the biological function of a gene, or a gene represented by an EST, and to validate the function of potential target genes for drug 10 discovery.

In one embodiment, expression of at least two different genes is regulated, using different zinc finger proteins to regulate each gene. One of the genes is a candidate gene, and the other gene can be a control gene or a second candidate gene. Cells expressing the genes are contacted with zinc finger proteins, or nucleic acids encoding 15 zinc finger proteins. Both the genes can be expressed in the same cell, or the genes can be each expressed in a different cell. After expression of the first and second genes is modulated by the zinc finger protein, the cells are assayed for changes in a selected phenotype, thereby identifying the function of the candidate gene or genes. In another embodiment, two zinc finger proteins target the same candidate gene at two different 20 target sites. The methods and compositions disclosed herein can be applied both to functional genomics, which typically refers to identifying genes associated with a particular phenotype, and for target validation, which typically refers to identifying genes that are suitable for use in drug discovery assays.

As a result, exogenous regulatory molecules such as, for example, zinc 25 finger proteins can be used to identify genes that cause a selected phenotype, both through activation and/or repression of gene transcription. Zinc finger proteins that bind to a promoter region can be used, but zinc finger proteins can also regulate gene expression by binding to other regions of the gene. Extensive sequence information is therefore not required to examine expression of a candidate gene using zinc finger 30 proteins. ESTs therefore can be used in the assays described herein, to determine their biological function.

0007.20 S7-US3

Furthermore, the zinc finger proteins can also be linked to regulatory domains, creating chimeric transcription factors to activate or repress transcription. In one embodiment, the methods of regulation use zinc finger proteins wherein the gene encoding the zinc finger protein is linked to molecular switches controlled by small molecules. The gene expression of the zinc finger proteins is therefore conditional and can be regulated using small molecules, thereby providing conditional regulation of candidate gene expression.

Such functional genomics assays allow for discovery of novel human and mammalian therapeutic applications, including the discovery of novel drugs, for, e.g.,

10 treatment of genetic diseases, cancer, fungal, protozoal, bacterial, and viral infection, ischemia, vascular disease, arthritis, immunological disorders, etc. Examples of assay systems for changes in phenotype include, e.g., transformation assays, e.g., changes in proliferation, anchorage dependence, growth factor dependence, foci formation, growth in soft agar, tumor proliferation in nude mice, and tumor vascularization in nude mice;

15 apoptosis assays, e.g., DNA laddering and cell death, expression of genes involved in apoptosis; signal transduction assays, e.g., changes in intracellular calcium, cAMP, cGMP, IP3, changes in hormone and neurotransmitter release; receptor assays, e.g., estrogen receptor and cell growth; growth factor assays, e.g., EPO, hypoxia and erythrocyte colony forming units assays; enzyme product assays, e.g., FAD-2 induced oil

20 desaturation; transcription assays, e.g., reporter gene assays; and protein production assays, e.g., VEGF ELISAs.

In one embodiment, a plurality of candidate genes is provided, and a first zinc finger protein is used to modulate expression of one of the candidate genes, while the expression pattern of the other candidate genes is examined. This step is repeated for

25 each of the candidate genes, and changes in the expression patterns are used to determine the biological function of the genes. The expression data can then be analyzed to reconstruct the order or cascade of genes in a pathway that is associated with a selected phenotype.

As described herein, zinc finger proteins can be designed to recognize any

30 suitable target site, for regulation of expression of any control or candidate gene of choice. Examples of target genes suitable for regulation include VEGF, CCR5, ER α ,

09/01/2010 09:28:00

Her2/Neu, Tat, Rev, HBV C, S, X, and P, LDL-R, PEPCK, CYP7, Fibrinogen, ApoB, Apo E, Apo(a), renin, NF- κ B, I- κ B, TNF- α , FAS ligand, amyloid precursor protein, atrial natriuretic factor, ob-leptin, ucp-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12, G-CSF, GM-CSF, Epo, PDGF, PAF, p53, Rb, fetal hemoglobin, dystrophin, eutrophin, GDNF, NGF,

5 IGF-1, VEGF receptors flt and flk, topoisomerase, telomerase, bcl-2, cyclins, angiostatin, IGF, ICAM-1, STATS, c-myc, c-myb, TH, PTI-1, polygalacturonase, EPSP synthase, FAD2-1, delta-12 desaturase, delta-9 desaturase, delta-15 desaturase, acetyl-CoA carboxylase, acyl-ACP-thioesterase, ADP-glucose pyrophosphorylase, starch synthase, cellulose synthase, sucrose synthase, senescence-associated genes, heavy metal chelators,

10 fatty acid hydroperoxide lyase, viral genes, protozoal genes, fungal genes, and bacterial genes. In general, suitable genes to be regulated include cytokines, lymphokines, growth factors, mitogenic factors, chemotactic factors, onco-active factors, receptors, potassium channels, G-proteins, signal transduction molecules, and other disease-related genes.

In a further embodiment, association between a gene and a selected phenotype (e.g., a biological function of a gene) is determined by assaying three types of cells. The first cell comprises a first exogenous molecule (e.g., a zinc finger protein) which binds to a first target site in the gene and activates expression of the gene. The second cell comprises a second exogenous molecule which binds to a second target site in the gene and represses expression of the gene. The first and second target sites can 20 comprise the same sequence, or they can comprise different sequences. In the third cell, expression of the gene is not modulated by an exogenous molecule. The first, second and third cells are each assayed for a selected phenotype, and the phenotypes of each of the cells are compared. A difference in phenotype between the first cell and the third cell, or between the second cell and the third cell, or between the first and second cells on the one 25 hand, and the third cell on the other, provides evidence for an association of the gene with the selected phenotype and, in many cases, indicates the biological function of a gene.

In a preferred embodiment, the first and second exogenous molecules each comprise a functional domain (e.g., a regulatory domain). In additional embodiments, either or both of the first and second exogenous molecules do not comprise a regulatory 30 domain. In certain embodiments, the first and second exogenous molecules comprise the same functional domain, which is a bifunctional domain whose activity is dependent on

100-1650

the presence of a second molecule such as, for example, a protein or small molecule.

(Said second molecule is distinct from, and not to be confused with, the second exogenous molecule described above.)

A cell can be subjected to one or more stimuli subsequent to contact with

- 5 an exogenous molecule and prior to assay for a selected phenotype. Such stimuli can include, but are not limited to, serum starvation, depletion of one or more external factors (e.g., ligands, growth factors), addition of one or more external factors (e.g., ligands, growth factors), stress (e.g., heat shock, cold shock, changes in pressure, hypoxia, anoxia, exposure to one or more oxidizing agents, exposure to one or more reducing agents,
- 10 exposure to one or more mutagens, exposure to one or more inhibitors of DNA synthesis or DNA repair, and exposure to one or more DNA damaging agents such a chemical or irradiation) and treatment of a cell with a compound. In addition, cells can be exposed to one or more pathogens (e.g., bacteria, viruses, unicellular eukaryotes) between contact with an exogenous molecule and assay for a selected phenotype, to determine whether
- 15 modulation of gene expression affects the response of the cell to the pathogen.

A selected phenotype can be any phenotype that can be detected by any method known in the art. In certain embodiments, the phenotype provides information on a biological function of a gene. Exemplary phenotypes include changes in cell physiology (e.g., energy metabolism, synthesis of cellular molecules, ion flux, membrane potential), changes in cellular morphology, changes in cell proliferation, changes in cell cycle properties (e.g., arrest at a particular stage in the cell cycle, unregulated cellular proliferation), changes in cellular metabolism (e.g., ATP levels, second messenger levels, cell transformation) and changes in any of the aforementioned properties that occur in response to exposure to a pathogen.

- 20
- 25

In a further embodiment, a cell can comprise an exogenous nucleic acid, which can encode a polypeptide, the expression of which can be connected with a cellular phenotype. In certain embodiments, the polypeptide is an endogenous polypeptide and the phenotype is correlated with overexpression of the endogenous polypeptide. In separate embodiments, the exogenous nucleic acid encodes a mutant form of an endogenous polypeptide, and the phenotype may, for example, mimic that of a mutation in the cellular gene encoding the polypeptide. In these embodiments, modulation of

- 30

expression (e.g., up- regulation and/or down-regulation) of a cellular gene, by contacting a cell with an exogenous molecule, can alter a phenotype resulting from expression of the exogenous nucleic acid in the cell, and the selected phenotype corresponds to said altered phenotype.

5 Candidate genes are selected by methods known to those of skill in the art, e.g., by gene expression microarrays, differential cDNA cloning frequencies, subtractive hybridization, differential display methods, by cloning ESTs from cells or tissues of interest, by identifying genes that are lethal upon knockout, by identifying genes that are up- or down-regulated in response to a particular developmental or cellular event or

10 stimuli; by identifying genes that are up- or down- regulated in certain disease and pathogenic states, by identifying mutations and RFLPs, by identifying genes associated with regions of chromosomes known to be involved in inherited diseases, by identifying genes that are temporally regulated, e.g., in a pathogenic organism, differences based on SNPs, etc.

15 A general theme in transcription factor function is that simple binding and, in some cases, sufficient proximity to the promoter are all that is generally needed. Exact positioning relative to the promoter, orientation, and within limits, distance do not matter greatly. In some cases enhancers are found positioned large distances away from the gene of interest. In addition, for repression of gene expression, often simple steric

20 hindrance of transcription initiation is sufficient. These features allow considerable flexibility in choosing target sites for zinc finger proteins. The target site recognized by the zinc finger protein therefore can be any suitable site in the target gene that will allow activation or repression of gene expression by a zinc finger protein, optionally linked to a regulatory domain. Preferred target sites include regions adjacent to, downstream, or

25 upstream of the transcription start site. In addition, target sites that are located in enhancer regions, repressor sites, RNA polymerase pause sites, and specific regulatory sites (e.g., SP-1 sites, hypoxia response elements, nuclear receptor recognition elements, p53 binding sites), sites in the cDNA encoding region or in an expressed sequence tag (EST) coding region. As described below, typically each finger recognizes 2-4 base

30 pairs, with a two finger zinc finger protein binding to a 4 to 7 bp target site, a three finger

zinc finger protein binding to a 6 to 10 base pair site, and a six finger zinc finger protein binding to two adjacent target sites, each target site having from 6-10 base pairs.

Recognition of adjacent target sites by either associated or individual zinc finger proteins can be used to produce enhanced binding of the zinc finger proteins, 5 resulting in an affinity that is greater than the affinity of the zinc finger proteins when individually bound to their target site. In one embodiment, a six finger zinc finger protein is produced as a fusion protein linked by an amino acid linker, and the resulting zinc finger protein recognizes an approximately 18 base pair target site (see, e.g., Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530 (1997)). An 18 base pair target site is 10 expected to provide specificity in the human genome, as a target site of that size should occur only once in every 3×10^{10} base pairs, and the size of the human genome is 3.5×10^9 base pairs (see, e.g., Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530 (1997)). In another embodiment, the two three-fingered portions of the six fingered zinc finger protein are non-covalently associated, through a leucine zipper, a STAT protein N- 15 terminal domain, or the FK506 binding protein (see, e.g., O'Shea, *Science* 254: 539 (1991), Barahmand-Pour *et al.*, *Curr. Top. Microbiol. Immunol.* 211:121-128 (1996); Klemm *et al.*, *Annu. Rev. Immunol.* 16:569-592 (1998); Ho *et al.*, *Nature* 382:822-826 (1996)).

As described herein, two zinc finger proteins are administered to a cell, 20 recognizing different target genes, e.g., a candidate gene and a control gene, or two candidate genes, or two different target sites for the same gene. Optionally, a plurality of zinc finger proteins can be administered, which recognize two or more different target sites in the same gene. When two candidate genes are examined, both the first and the second gene may be required for the phenotype. The candidate genes may be endogenous 25 genes or exogenous genes. In one embodiment, more than one candidate gene is associated with a selected phenotype.

In another embodiment, the zinc finger protein is linked to at least one or more regulatory domains, described below. Preferred regulatory domains include transcription factor repressor or activator domains such as KRAB and VP16, co-repressor 30 and co-activator domains, DNA methyl transferases, histone acetyltransferases, histone deacetylases, and endonucleases such as Fok1. For repression of gene expression,

T00290509760

typically the expression of the gene is reduced by about 20% (i.e., 80% of non-zinc finger protein modulated expression), more preferably by about 50% (i.e., 50% of non-zinc finger protein modulated expression), more preferably by about 75-100% (i.e., 25% to 0% of non-zinc finger protein modulated expression). For activation of gene expression,
5 typically expression is activated by about 1.5 fold (i.e., 150% of non-zinc finger protein modulated expression), preferably 2 fold (i.e., 200% of non-zinc finger protein modulated expression), more preferably 5-10 fold (i.e., 500-1000% of non-zinc finger protein modulated expression), up to at least 100 fold or more.

The expression of engineered zinc finger protein activators and repressors
10 can be also controlled by small molecule systems typified by the tet-regulated systems and the RU-486 system (see, e.g., Gossen & Bujard, *Proc. Natl. Acad. Sci. U.S.A.* 89:5547 (1992); Oligino *et al.*, *Gene Ther.* 5:491-496 (1998); Wang *et al.*, *Gene Ther.* 4:432-441 (1997); Neering *et al.*, *Blood* 88:1147-1155 (1996); and Rendahl *et al.*, *Nat. Biotechnol.* 16:757-761 (1998)). These impart small molecule control on the expression
15 of the zinc finger protein activators and repressors and thus impart small molecule control on the target gene(s) of interest. This beneficial feature could be used in cell culture models, and in transgenic animals and plants.

The practice of the disclosed methods and use of the disclosures
20 compositions employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, genetics, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. See, for example, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; and the series METHODS IN ENZYMOLOGY, Academic Press, San Diego.

The disclosures of all patents, patent applications and publications mentioned herein are hereby incorporated by reference in their entireties.

Definitions

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

A “candidate gene” refers to a cellular, viral, episomal, microbial,

5 protozoal, fungal, animal, plant, chloroplastic, or mitochondrial gene. This term also refers to a microbial or viral gene that is part of a naturally occurring microbial or viral genome in a microbially or virally infected cell. The microbial or viral genome can be extrachromosomal or integrated into the host chromosome. This term also encompasses endogenous and exogenous genes, as well as cellular genes that are identified as ESTs.

10 Often, candidate genes are those for which the biological function is unknown. An assay of choice is used to determine whether or not the gene is associated with a selected phenotype upon regulation of candidate gene expression with a zinc finger protein. If the biological function is known, typically the candidate gene acts as a control gene, or is used to determine if one or more additional genes are associated with the same

15 phenotype, or is used to determine if the gene participates with other genes in a particular phenotype.

A “gene,” for the purposes of the present disclosure, includes a DNA region encoding a gene product (see below), as well as all DNA regions which regulate the production of the gene product, including regulatory regions, promoters, and enhancers.

20 to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, coding region (*i.e.* nucleotide sequences encoding the amino acid sequence of a polypeptide gene product); transcribed region (*i.e.*, nucleotide sequences serving as template for transcription of a RNA molecule); nontranscribed regulatory regions such as, for example, promoter sequences, transcription start sites, and
25 transcription termination sites; translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites; enhancers; silencers; insulators; boundary elements; replication origins; matrix attachment sites and locus control regions. Further, a promoter can be a cellular promoter or a promoter of an infecting microorganism such as, for example, a virus, bacterium or unicellular eukaryote. A gene can be a cellular
30 gene of, for example, a plant, animal or fungus, or a gene can be part of the genome of an infectious agent such as, for example, a virus, bacterium, or unicellular eukaryote.

Gene expression” refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, enzymatic RNA (e.g., ribozyme), structural RNA, regulatory RNA or any other type of RNA) or a protein produced by 5 translation of a mRNA encoded by a gene. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for 10 example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

“Gene activation” and “augmentation of gene expression” refer to any process which results in an increase in production of a gene product. A gene product can be either RNA (including, but not limited to, mRNA, rRNA, tRNA, snoRNA, snRNA, 15 telomerase RNA, 7SL signal recognition particle RNA, structural RNA, regulatory RNA, enzymatic RNA) or protein. Accordingly, gene activation includes those processes which increase transcription of a gene and/or translation of a mRNA. Examples of gene activation processes which increase transcription include, but are not limited to, those which facilitate formation of a transcription initiation complex, those which increase 20 transcription initiation rate, those which increase transcription elongation rate, those which increase processivity of transcription and those which relieve transcriptional repression (by, for example, blocking the binding of a transcriptional repressor). Gene activation can constitute, for example, inhibition of repression as well as stimulation of expression above an existing level. Examples of gene activation processes which 25 increase translation include those which increase translational initiation, those which increase translational elongation and those which increase mRNA stability. In general, gene activation comprises any detectable increase in the production of a gene product, preferably an increase in production of a gene product by about 2-fold, more preferably from about 2- to about 5-fold or any integral value therebetween, more preferably 30 between about 5- and about 10-fold or any integral value therebetween, more preferably between about 10- and about 20-fold or any integral value therebetween, still more

100-0007.20 S7-US3

preferably between about 20- and about 50-fold or any integral value therebetween, more preferably between about 50- and about 100-fold or any integral value therebetween, more preferably 100-fold or more.

“Gene repression” and “inhibition of gene expression” refer to any process which results in a decrease in production of a gene product. A gene product can be either RNA (including, but not limited to, mRNA, rRNA, tRNA, snoRNA, snRNA, telomerase RNA, 7SL signal recognition particle RNA, structural RNA, regulatory RNA, enzymatic RNA) or protein. Accordingly, gene repression includes those processes which decrease transcription of a gene and/or translation of a mRNA. Examples of gene repression processes which decrease transcription include, but are not limited to, those which inhibit formation of a transcription initiation complex, those which decrease transcription initiation rate, those which decrease transcription elongation rate, those which decrease processivity of transcription and those which antagonize transcriptional activation (by, for example, blocking the binding of a transcriptional activator). Gene repression can constitute, for example, prevention of activation as well as inhibition of expression below an existing level. Examples of gene repression processes which decrease translation include those which decrease translational initiation, those which decrease translational elongation and those which decrease mRNA stability. Transcriptional repression includes both reversible and irreversible inactivation of gene transcription. In general, gene repression comprises any detectable decrease in the production of a gene product, preferably a decrease in production of a gene product by about 2-fold, more preferably from about 2- to about 5-fold or any integral value therebetween, more preferably between about 5- and about 10-fold or any integral value therebetween, more preferably between about 10- and about 20-fold or any integral value therebetween, still more preferably between about 20- and about 50-fold or any integral value therebetween, more preferably between about 50- and about 100-fold or any integral value therebetween, more preferably 100-fold or more. Most preferably, gene repression results in complete inhibition of gene expression, such that no gene product is detectable.

The term “modulate” refers to a change in the quantity, degree or extent of a function. For example, exogenous molecules such as zinc finger proteins can modulate gene expression by binding to a target sequence within or outside of a gene, thereby

2025 0007.20 S7-US3

inducing, enhancing or suppressing transcription of the gene. In addition, modulation can include inhibition of transcription of a gene wherein the modified zinc finger-nucleotide binding polypeptide binds to the transcribed region of a gene and blocks the passage of DNA dependent RNA polymerase, thus inhibiting transcription of the gene. Furthermore, 5 modulation can include stimulation or inhibition of translation of a transcript. Thus, “modulation” of gene expression can occur through effects on both DNA and RNA and includes both activation and repression of gene expression.

Accordingly, the terms “modulating expression” “inhibiting expression” and “activating expression” of a gene can refer to the ability of a zinc finger protein to 10 activate or inhibit transcription of a gene. Activation includes prevention of transcriptional inhibition (i.e., prevention of repression of gene expression) and inhibition includes prevention of transcriptional activation (i.e., prevention of gene activation).

“Activation of gene expression that prevents repression of gene expression” refers to the ability of a zinc finger protein to block or prevent binding of a 15 repressor molecule.

“Inhibition of gene expression that prevents gene activation” refers to the ability of a zinc finger protein to block or prevent binding of an activator molecule.

Modulation can be assayed by determining any parameter that is indirectly or directly affected by the expression of the target gene. Such parameters include, e.g., 20 changes in RNA or protein levels; changes in protein activity; changes in product levels; changes in downstream gene expression; changes in transcription or activity of reporter genes such as, for example, luciferase, CAT, beta-galactosidase, or GFP (see, e.g., Mistili & Spector, (1997) *Nature Biotechnology* 15:961-964); changes in signal transduction; changes in phosphorylation and dephosphorylation; changes in receptor-ligand 25 interactions; changes in concentrations of second messengers such as, for example, cGMP, cAMP, IP₃, and Ca²⁺; changes in cell growth, changes in neovascularization, and/or changes in any functional effect of gene expression. Measurements can be made *in vitro*, *in vivo*, and/or *ex vivo*. Such functional effects can be measured by conventional 30 methods, e.g., measurement of RNA or protein levels, measurement of RNA stability, and/or identification of downstream or reporter gene expression. Readout can be by way of, for example, chemiluminescence, fluorescence, colorimetric reactions, antibody

CONFIDENTIAL - 082650

binding, inducible markers, ligand binding assays; changes in intracellular second messengers such as cGMP and inositol triphosphate (IP_3); changes in intracellular calcium levels; cytokine release, and the like.

To determine the level of gene expression modulation by a zinc finger protein, cells contacted with zinc finger proteins are compared to control cells, e.g., without the zinc finger protein or with a non-specific zinc finger protein, to examine the extent of inhibition or activation. Control samples are assigned a relative gene expression activity value of 100%. Modulation/inhibition of gene expression is achieved when the gene expression activity value relative to the control is about 80%, preferably 50% (i.e., 0.5x the activity of the control), more preferably 25%, more preferably 5-0%. Modulation/activation of gene expression is achieved when the gene expression activity value relative to the control is 110% , more preferably 150% (i.e., 1.5x the activity of the control), more preferably 200-500%, more preferably 1000-2000% or more.

An “exogenous molecule” is a molecule that is not normally present in a cell, but can be introduced into a cell by one or more genetic, biochemical or other methods. Normal presence in the cell is determined with respect to the particular developmental stage and environmental conditions of the cell. Thus, for example, a molecule that is present only during embryonic development of muscle is an exogenous molecule with respect to an adult muscle cell. Similarly, a molecule induced by heat shock is an exogenous molecule with respect to a non-heat-shocked cell. An exogenous molecule can comprise, for example, a functioning version of a malfunctioning endogenous molecule or a malfunctioning version of a normally-functioning endogenous molecule.

An exogenous molecule can be, among other things, a small molecule, such as is generated by a combinatorial chemistry process, or a macromolecule such as a protein, nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein, polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Nucleic acids include DNA and RNA, can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Nucleic acids include those capable of forming duplexes, as well as triplex-forming nucleic acids. See, for example, U.S. Patent Nos. 5,176,996 and 5,422,251. Proteins include, but are not

limited to, DNA-binding proteins, transcription factors, chromatin remodeling factors, methylated DNA binding proteins, polymerases, methylases, demethylases, acetylases, deacetylases, kinases, phosphatases, integrases, recombinases, ligases, topoisomerases, gyrases and helicases.

5 An exogenous molecule can be the same type of molecule as an endogenous molecule, *e.g.*, protein or nucleic acid (*i.e.*, an exogenous gene), providing it has a sequence that is different from an endogenous molecule. For example, an exogenous nucleic acid can comprise an infecting viral genome, a plasmid or episome introduced into a cell, or a chromosome that is not normally present in the cell. Methods 10 for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.*, liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector-mediated transfer.

15 By contrast, an “endogenous molecule” is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, chloroplast or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include endogenous genes 20 and endogenous proteins, for example, transcription factors and components of chromatin remodeling complexes.

25 A “selected phenotype” refers to any phenotype, *e.g.*, any observable characteristic or functional effect that can be measured in an assay such as changes in cell growth, proliferation, morphology, enzyme function, signal transduction, expression patterns, downstream expression patterns, reporter gene activation, hormone release, growth factor release, neurotransmitter release, ligand binding, apoptosis, and product formation. Such assays include, *e.g.*, transformation assays, *e.g.*, changes in proliferation, anchorage dependence, growth factor dependence, foci formation, growth in soft agar, tumor proliferation in nude mice, and tumor vascularization in nude mice; apoptosis 30 assays, *e.g.*, DNA laddering and cell death, expression of genes involved in apoptosis; signal transduction assays, *e.g.*, changes in intracellular calcium, cAMP, cGMP, IP3,

10 15 20 25 30

changes in hormone and neurotransmitter release; receptor assays, e.g., estrogen receptor and cell growth; growth factor assays, e.g., EPO, hypoxia and erythrocyte colony forming units assays; enzyme product assays, e.g., FAD-2 induced oil desaturation; transcription assays, e.g., reporter gene assays; and protein production assays, e.g., VEGF ELISAs.

5 A candidate gene is “associated with” a selected phenotype if modulation of gene expression of the candidate gene causes a change in the selected phenotype.

The term “zinc finger protein” or “ZFP” refers to a protein having DNA binding domains that are stabilized by zinc. The individual DNA binding domains are typically referred to as “fingers” A zinc finger protein has least one finger, typically two 10 fingers, three fingers, or six fingers. Each finger binds from two to four base pairs of DNA, typically three or four base pairs of DNA. A zinc finger protein binds to a nucleic acid sequence called a target site or target segment. Each finger typically comprises an approximately 30 amino acid, zinc-coordinating, DNA-binding subdomain. An exemplary motif characterizing one class of these proteins (Cys₂His₂ class) is -Cys-(X)₂₋₄-Cys-(X)₁₂-His-(X)₃₋₅-His (SEQ ID NO: 1) (where X is any amino acid). Studies have 15 demonstrated that a single zinc finger of this class consists of an alpha helix containing the two invariant histidine residues co-ordinated with zinc along with the two cysteine residues of a single beta turn (see, e.g., Berg & Shi, *Science* 271:1081-1085 (1996)).

A “target site” is the nucleic acid sequence recognized by a zinc finger 20 protein. A single target site typically has about four to about ten base pairs. Typically, a two-fingered zinc finger protein recognizes a four to seven base pair target site, a three-fingered zinc finger protein recognizes a six to ten base pair target site, and a six fingered zinc finger protein recognizes two adjacent nine to ten base pair target sites.

The term “adjacent target sites” refers to non-overlapping target sites that 25 are separated by zero to about 5 base pairs.

“K_d” refers to the dissociation constant for the compound, i.e., the concentration of a compound (e.g., a zinc finger protein) that gives half maximal binding of the compound to its target (i.e., half of the compound molecules are bound to the target) under given conditions (i.e., when [target] << K_d), as measured using a given assay 30 system (see, e.g., U.S. Patent No. 5,789,538). The assay system used to measure the K_d should be chosen so that it gives the most accurate measure of the actual K_d of the zinc

TO BE MADE PUBLIC

finger protein. Any assay system can be used, as long as it gives an accurate measurement of the actual K_d of the zinc finger protein. In one embodiment, the K_d for a zinc finger protein is measured using an electrophoretic mobility shift assay (“EMSA”), as described herein. Unless an adjustment is made for zinc finger protein purity or activity, the K_d 5 calculations made using the methods described herein may result in an underestimate of the true K_d of a given zinc finger protein. Optionally, the K_d of a zinc finger protein used to modulate transcription of a candidate gene is less than about 100 nM, or less than about 75 nM, or less than about 50 nM, or less than about 25 nM.

The phrase “adjacent to a transcription initiation site” refers to a target site 10 that is within about 50 bases either upstream or downstream of a transcription initiation site. “Upstream” of a transcription initiation site refers to a target site that is more than about 50 bases 5’ of the transcription initiation site. “Downstream” of a transcription initiation site refers to a target site that is more than about 50 bases 3’ of the transcription initiation site.

15 The phrase “RNA polymerase pause site” is described in Uptain *et al.*, *Annu. Rev. Biochem.* 66:117-172 (1997).

“Administering” an expression vector, nucleic acid, zinc finger protein, or 20 a delivery vehicle to a cell comprises transducing, transfecting, electroporating, translocating, fusing, phagocytosing, or biolistic methods, etc., i.e., any means by which a protein or nucleic acid can be transported across a cell membrane and preferably into the nucleus of a cell, including administration of naked DNA.

A “delivery vehicle” refers to a compound, e.g., a liposome, toxin, or a 25 membrane translocation polypeptide, which is used to administer a zinc finger protein. Delivery vehicles can also be used to administer nucleic acids encoding zinc finger proteins, e.g., a lipid:nucleic acid complex, an expression vector, a virus, and the like.

A “transcriptional activator” and a “transcriptional repressor” refer to 30 proteins or functional fragments of proteins that have the ability to modulate transcription, as described above. Such proteins include, e.g., transcription factors and co-factors (e.g., KRAB, MAD, ERD, SID, nuclear factor kappa B subunit p65, early growth response factor 1, and nuclear hormone receptors, VP16, VP64), endonucleases, integrases, recombinases, methyltransferases, histone acetyltransferases, histone

deacetylases etc. Activators and repressors include co-activators and co-repressors (see, e.g., Utley *et al.*, *Nature* 394:498-502 (1998)).

A “regulatory domain” or “functional domain” refers to a protein or a polypeptide sequence that has transcriptional modulation activity, or that is capable of interacting with proteins and/or protein domains that have transcriptional modulation activity. Typically, a functional domain is covalently or non-covalently linked to a DNA-binding domain (e.g., a ZFP) to modulate transcription of a gene of interest. Alternatively, a ZFP can act, in the absence of a functional domain, to modulate transcription. Furthermore, transcription of a gene of interest can be modulated by a ZFP linked to multiple functional domains.

A “functional fragment” of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length protein, polypeptide or nucleic acid, yet retains the same function as the full-length protein, polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same number of residues as the corresponding native molecule, and/or can contain one or more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (e.g., coding function, ability to hybridize to another nucleic acid, binding to a regulatory molecule) are well-known in the art. Similarly, methods for determining protein function are well-known. For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. See Ausubel *et al.*, *supra*. The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and biochemical. See, for example, Fields *et al.* (1989) *Nature* 340:245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

A “fusion molecule” is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion polypeptides (for example, a fusion between a ZFP DNA-binding domain and a functional domain) and fusion nucleic acids (for example, a nucleic acid encoding a fusion polypeptide).

Examples of the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

The term “heterologous” is a relative term, which when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, a nucleic acid that is recombinantly produced typically has two or more sequences from unrelated genes synthetically arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. The two nucleic acids are thus heterologous to each other in this context. When added to a cell, the recombinant nucleic acids would also be heterologous to the endogenous genes of the cell. Thus, in a chromosome, a heterologous nucleic acid would include an non-native (non-naturally occurring) nucleic acid that has integrated into the chromosome, or a non-native (non-naturally occurring) extrachromosomal nucleic acid.

Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a “fusion protein,” where the two subsequences are encoded by a single nucleic acid sequence). *See, e.g., Ausubel, supra*, for an introduction to recombinant techniques.

The term “recombinant” when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (naturally occurring) form of the cell or express a second copy of a native gene that is otherwise normally or abnormally expressed, under expressed or not expressed at all.

A “promoter” is defined as an array of nucleic acid control sequences that direct transcription. As used herein, a promoter typically includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of certain RNA polymerase II type promoters, a TATA element, enhancer, CCAAT box, SP-1 site, etc. As used herein, a promoter also optionally includes distal enhancer or repressor elements,

which can be located as much as several thousand base pairs from the start site of transcription. The promoters often have an element that is responsive to transactivation by a DNA-binding moiety such as a polypeptide, e.g., a nuclear receptor, Gal4, the lac repressor and the like.

5 A “constitutive” promoter is a promoter that is active under most environmental and developmental conditions. An “inducible” promoter is a promoter that is active under certain environmental or developmental conditions.

Nucleic acid or amino acid sequences are “operably linked” (or “operatively linked”) when placed into a functional relationship with one another. For instance, a promoter or enhancer is operably linked to a coding sequence if it regulates, or contributes to the modulation of, the transcription of the coding sequence. Operably linked DNA sequences are typically contiguous, and operably linked amino acid sequences are typically contiguous and in the same reading frame. However, since enhancers generally function when separated from the promoter by up to several kilobases or more and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous. Similarly, certain amino acid sequences that are non-contiguous in a primary polypeptide sequence may nonetheless be operably linked due to, for example folding of a polypeptide chain.

With respect to fusion polypeptides, the terms “operatively linked” and “operably linked” can refer to the fact that each of the components performs the same function in linkage to the other component as it would if it were not so linked. For example, with respect to a fusion polypeptide in which a ZFP DNA-binding domain is fused to a transcriptional activation domain (or functional fragment thereof), the ZFP DNA-binding domain and the transcriptional activation domain (or functional fragment thereof) are in operative linkage if, in the fusion polypeptide, the ZFP DNA-binding domain portion is able to bind its target site and/or its binding site, while the transcriptional activation domain (or functional fragment thereof) is able to activate transcription.

An “expression vector” is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell, and optionally, integration or replication of the expression vector in a host cell. The expression vector can be part of

a plasmid, virus, or nucleic acid fragment, of viral or non-viral origin. Typically, the expression vector includes an “expression cassette,” which comprises a nucleic acid to be transcribed operably linked to a promoter. The term expression vector also encompasses naked DNA operably linked to a promoter.

5 By "host cell" is meant a cell that contains a ZFP or an expression vector or nucleic acid encoding a ZFP. The host cell typically supports the replication or expression of the expression vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as fungal cells (e.g., yeast), protozoal cells, plant cells, insect cells, animal cells, avian cells, teleost cells, amphibian cells, mammalian cells, primate cells or human cells.

10 Exemplary mammalian cell lines include CHO, HeLa, 293, COS-1, and the like, e.g., cultured cells (*in vitro*), explants and primary cultures (*in vitro* and *ex vivo*), and cells *in vivo*.

“Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a

corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill

PROVISIONAL DRAFT

will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles.

The following eight groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- 7) Serine (S), Threonine (T); and
- 8) Cysteine (C), Methionine (M)

(*see, e.g.*, Creighton, *Proteins* (1984)).

The transcriptional program of the cell or "transcriptome" refers to a collection of mRNA molecules present in a given cell under a given set of environmental conditions, and can be determined by methods known to those of skill in the art, such as, for example, microarray analysis, serial analysis of gene expression, and mRNA or cDNA display techniques. *See* for example, US Patents No. 5,599,672 and 5,695,937.

Environmental conditions can include, but are not limited to, the tissue or culture medium in which the cell resides, stage of development, disease state, infection and conditions

such as, for example, temperature, pressure and the presence of one or more extracellular ligands, mitogens or growth factors, for example. A transcriptome can be complete (*i.e.*, it can include all mRNA molecules present in a cell) or it can be partial such as, for example, when analysis is limited to just those mRNAs which can be detected with a 5 particular microarray. Additional transcriptomal information can include relative and/or absolute levels for each mRNA in the transcriptome. Differences between the transcriptomes of two or more cells can be determined by methods known to those of skill in the art including, but not limited to subtractive hybridization and related types of difference analysis, differential mRNA or cDNA display, serial analysis of gene 10 expression and microarray analysis. *See*, for example, US Patents No. 5,436,142; 5,501,964; 5,958,738; 5,665,547; 5,965,409; and 5,695,937.

Design of zinc finger proteins

Exogenous regulatory molecules (*e.g.*, zinc finger proteins) are engineered 15 to recognize a selected target site in the candidate gene of choice. Typically, a backbone from any suitable Cys₂His₂ zinc finger protein, such as SP-1, SP-1C, or ZIF268, is used as the scaffold for the engineered zinc finger protein (*see, e.g.*, Jacobs, *EMBO J.* 11:4507 (1992); Desjarlais & Berg, *Proc. Natl. Acad. Sci. U.S.A.* 90:2256-2260 (1993)). A 20 number of methods can then be used to design and select a zinc finger protein with high affinity for its target (*e.g.*, preferably with a K_d of less than about 25 nM). As described above, a zinc finger protein can be designed or selected to bind to any suitable target site in the target candidate gene, with high affinity. Co-owned PCT WO 00/42219 (herein incorporated by reference in its entirety), comprehensively describes methods for design, construction, and expression of zinc finger proteins for selected target sites.

25 Any suitable method known in the art can be used to design and construct nucleic acids encoding zinc finger proteins, *e.g.*, phage display, random mutagenesis, combinatorial libraries, computer/rational design, affinity selection, PCR, cloning from cDNA or genomic libraries, synthetic construction and the like. (*see, e.g.*, U.S. Pat. No. 5,786,538; Wu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:344-348 (1995); Jamieson *et al.*, 30 *Biochemistry* 33:5689-5695 (1994); Rebar & Pabo, *Science* 263:671-673 (1994); Choo & Klug, *Proc. Natl. Acad. Sci. U.S.A.* 91:11163-11167 (1994); Choo & Klug, *Proc. Natl.*

Acad. Sci. U.S.A. 91: 11168-11172 (1994); Desjarlais & Berg, *Proc. Natl. Acad. Sci. U.S.A.* 90:2256-2260 (1993); Desjarlais & Berg, *Proc. Natl. Acad. Sci. U.S.A.* 89:7345-7349 (1992); Pomerantz *et al.*, *Science* 267:93-96 (1995); Pomerantz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:9752-9756 (1995); and Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530 (1997); Greisman & Pabo, *Science* 275:657-661 (1997); Desjarlais & Berg, *Proc. Natl. Acad. Sci. U.S.A.* 91:11-99-11103 (1994)).

In a preferred embodiment, co-owned PCT WO 00/42219 provides methods that select a target gene, and identify a target site within the gene containing one to six (or more) D-able sites (see definition below). Using these methods, a zinc finger protein can then be synthesized that binds to the preselected site. These methods of target site selection are premised, in part, on the recognition that the presence of one or more D-able sites in a target segment confers the potential for higher binding affinity in a zinc finger protein selected or designed to bind to that site relative to zinc finger proteins that bind to target segments lacking D-able sites. Experimental evidence supporting this insight is provided in Examples 2-9 of co-owned PCT WO 00/42219.

A D-able site or subsite is a region of a target site that allows an appropriately designed single zinc finger to bind to four bases rather than three of the target site. Such a zinc finger binds to a triplet of bases on one strand of a double-stranded target segment (target strand) and a fourth base on the other strand (see Figure 2 of co-owned PCT WO 00/42219. Binding of a single zinc finger to a four base target segment imposes constraints both on the sequence of the target strand and on the amino acid sequence of the zinc finger. The target site within the target strand should include the “D-able” site motif 5’ NNGK 3’, in which N and K are conventional IUPAC-IUB ambiguity codes. A zinc finger for binding to such a site should include an arginine residue at position -1 and an aspartic acid, (or less preferably a glutamic acid) at position +2. The arginine residues at position -1 interacts with the G residue in the D-able site. The aspartic acid (or glutamic acid) residue at position +2 of the zinc finger interacts with the opposite strand base complementary to the K base in the D-able site. It is the interaction between aspartic acid (symbol D) and the opposite strand base (fourth base) that confers the name D-able site. As is apparent from the D-able site formula, there are two subtypes of D-able sites: 5’ NNGG 3’ and 5’ NNGT 3’. For the former site, the

PCT/EP00/026650

aspartic acid or glutamic acid at position +2 of a zinc finger interacts with a C in the opposite strand to the D-able site. In the latter site, the aspartic acid or glutamic acid at position +2 of a zinc finger interacts with an A in the opposite strand to the D-able site. In general, NNGG is preferred over NNGT.

5 In the design of a zinc finger protein with three fingers, a target site should be selected in which at least one finger of the protein, and optionally, two or all three fingers have the potential to bind a D-able site. Such can be achieved by selecting a target site from within a larger target gene having the formula 5'-NNx aNy bNzc-3', wherein

10

each of the sets (x, a), (y, b) and (z, c) is either (N, N) or (G, K);
at least one of (x, a), (y, b) and (z, c) is (G, K). and
N and K are IUPAC-IUB ambiguity codes

15

In other words, at least one of the three sets (x, a), (y, b) and (z, c) is the set (G, K), meaning that the first position of the set is G and the second position is G or T. Those of the three sets (if any) which are not (G, K) are (N, N), meaning that the first position of the set can be occupied by any nucleotide and the second position of the set can be occupied by any nucleotide. As an example, the set (x, a) can be (G, K) and the sets (y, b) and (z, c) can both be (N, N).

20

In the formula 5'-NNx aNy bNzc-3', the triplets of NNx aNy and bNzc represent the triplets of bases on the target strand bound by the three fingers in a zinc finger protein. If only one of x, y and z is a G, and this G is followed by a K, the target site includes a single D-able subsite. For example, if only x is G, and a is K, the site reads 5'-**NNG** KNy bNzc-3' with the D-able subsite highlighted. If both x and y but not z are G, and a and b are K, then the target site has two overlapping D-able subsites as follows: 5'-**NNG** *KNG* KNz c-3' (SEQ ID NO: 2), with one such site being represented in bold and the other in italics. If all three of x, y and z are G and a, b, and c are K, then the target segment includes three D-able subsites, as follows 5'**NNG** *KNG* KNG K3' (SEQ ID NO: 3), the D-able subsites being represented by bold, italics and underline.

TIBETAN - CHINESE

These methods thus work by selecting a target gene, and systematically searching within the possible subsequences of the gene for target sites conforming to the formula 5'-NNx aNy bNzc-3', as described above. In some such methods, every possible subsequence of 10 contiguous bases on either strand of a potential target gene is evaluated 5 to determine whether it conforms to the above formula, and, if so, how many D-able sites are present. Typically, such a comparison is performed by computer, and a list of target sites conforming to the formula are output. Optionally, such target sites can be output in different subsets according to how many D-able sites are present.

In a variation, the methods identify first and second target segments, each 10 independently conforming to the above formula. The two target segments in such methods are constrained to be adjacent or proximate (i.e., within about 0-5 bases) of each other in the target gene. The strategy underlying selection of proximate target segments is to allow the design of a zinc finger protein formed by linkage of two component zinc finger proteins specific for the first and second target segments respectively. These 15 principles can be extended to select target sites to be bound by zinc finger proteins with any number of component fingers. For example, a suitable target site for a nine finger protein would have three component segments, each conforming to the above formula.

The target sites identified by the above methods can be subject to further evaluation by other criteria or can be used directly for design or selection (if needed) and 20 production of a zinc finger protein specific for such a site. A further criteria for evaluating potential target sites is their proximity to particular regions within a gene. If a zinc finger protein is to be used to repress a cellular gene on its own (i.e., without linking the zinc finger protein to a repressing moiety), then the optimal location appears to be at, or within 50 bp upstream or downstream of the site of transcription initiation, to interfere 25 with the formation of the transcription complex (Kim & Pabo, *J. Biol. Chem.* 272:29795-296800 (1997)) or compete for an essential enhancer binding protein. If, however, a zinc finger protein is fused to a functional domain such as the KRAB repressor domain or the VP16 activator domain, the location of the binding site is considerably more flexible and can be outside known regulatory regions. For example, a KRAB domain can repress 30 transcription at a promoter up to at least 3 kbp from where KRAB is bound (Margolin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 91:4509-4513 (1994)). Thus, target sites can be selected

100-280-057560

that do not necessarily include or overlap segments of demonstrable biological significance with target genes, such as regulatory sequences. Other criteria for further evaluating target segments include the prior availability of zinc finger proteins binding to such segments or related segments, and/or ease of designing new zinc finger proteins to bind a given target segment.

After a target segment has been selected, a zinc finger protein that binds to the segment can be provided by a variety of approaches. The simplest of approaches is to provide a precharacterized zinc finger protein from an existing collection that is already known to bind to the target site. However, in many instances, such zinc finger proteins do not exist. An alternative approach can also be used to design new zinc finger proteins, which uses the information in a database of existing zinc finger proteins and their respective binding affinities. A further approach is to design a zinc finger protein based on substitution rules as discussed above. A still further alternative is to select a zinc finger protein with specificity for a given target by an empirical process such as phage display. In some such methods, each component finger of a zinc finger protein is designed or selected independently of other component fingers. For example, each finger can be obtained from a different preexisting zinc finger protein or each finger can be subject to separate randomization and selection.

Once a zinc finger protein has been selected, designed, or otherwise provided to a given target segment, the zinc finger protein or the DNA encoding it are synthesized. Exemplary methods for synthesizing and expressing DNA encoding zinc finger proteins are described below. The zinc finger protein or a polynucleotide encoding it can then be used for modulation of expression, or analysis of the target gene containing the target site to which the zinc finger protein binds.

25

Expression and purification of zinc finger proteins

Zinc finger protein polypeptides and nucleic acids can be made using routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods in the field include Sambrook *et al.*, *Molecular Cloning. A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)). In addition,

T002001054460

essentially any nucleic acid can be custom ordered from any of a variety of commercial sources. Similarly, peptides and antibodies can be custom ordered from any of a variety of commercial sources.

Two alternative methods are typically used to create the coding sequences required to express newly designed DNA-binding peptides. One protocol is a PCR-based assembly procedure that utilizes six overlapping oligonucleotides (see Figure 1 of co-owned PCT WO 00/41566). Three oligonucleotides correspond to “universal” sequences that encode portions of the DNA-binding domain between the recognition helices. These oligonucleotides remain constant for all zinc finger constructs. The other three “specific” oligonucleotides are designed to encode the recognition helices. These oligonucleotides contain substitutions primarily at positions -1, 2, 3 and 6 on the recognition helices making them specific for each of the different DNA-binding domains.

The PCR synthesis is carried out in two steps. First, a double stranded DNA template is created by combining the six oligonucleotides (three universal, three specific) in a four cycle PCR reaction with a low temperature annealing step, thereby annealing the oligonucleotides to form a DNA “scaffold.” The gaps in the scaffold are filled in by high-fidelity thermostable polymerase, the combination of Taq and Pfu polymerases also suffices. In the second phase of construction, the zinc finger template is amplified by external primers designed to incorporate restriction sites at either end for cloning into a shuttle vector or directly into an expression vector.

An alternative method of cloning the newly designed DNA-binding proteins relies on annealing complementary oligonucleotides encoding the specific regions of the desired zinc finger protein. This particular application requires that the oligonucleotides be phosphorylated prior to the final ligation step. This is usually performed before setting up the annealing reactions, but kinasing can also occur post-annealing. In brief, the “universal” oligonucleotides encoding the constant regions of the proteins are annealed with their complementary oligonucleotides. Additionally, the “specific” oligonucleotides encoding the finger recognition helices are annealed with their respective complementary oligonucleotides. These complementary oligos are designed to fill in the region which was previously filled in by polymerase in the protocol described above. The complementary oligos to the common oligos 1 and finger 3 are engineered to

100-0007-00000-00000

leave overhanging sequences specific for the restriction sites used in cloning into the vector of choice. The second assembly protocol differs from the initial protocol in the following aspects: the “scaffold” encoding the newly designed zinc finger protein is composed entirely of synthetic DNA thereby eliminating the polymerase fill-in step,
5 additionally the fragment to be cloned into the vector does not require amplification. Lastly, the design of leaving sequence-specific overhangs eliminates the need for restriction enzyme digests of the inserting fragment.

The resulting fragment encoding the newly designed zinc finger protein is ligated into an expression vector. Expression vectors that are commonly utilized include,
10 but are not limited to, a modified pMAL-c2 bacterial expression vector (New England BioLabs, “NEB”) or a eukaryotic expression vector, pcDNA (Promega).

Any suitable method of protein purification known to those of skill in the art can be used to purify zinc finger proteins (*see* Ausubel, *supra*, Sambrook, *supra*). In addition, any suitable host can be used, e.g., bacterial cells, insect cells, yeast cells,
15 mammalian cells, and the like.

In one embodiment, expression of the zinc finger protein fused to a maltose binding protein (MBP-ZFP) in bacterial strain JM109 allows for straightforward purification through an amylose column (NEB). High expression levels of the zinc finger chimeric protein can be obtained by induction with IPTG since the MBP-ZFP fusion in
20 the pMal-c2 expression plasmid is under the control of the IPTG inducible tac promoter (NEB). Bacteria containing the MBP-ZFP fusion plasmids are inoculated in to 2x YT medium containing 10 μ M ZnCl₂, 0.02% glucose, plus 50 μ g/ml ampicillin and shaken at 37°C. At mid-exponential growth IPTG is added to 0.3 mM and the cultures are allowed to shake. After 3 hours the bacteria are harvested by centrifugation, disrupted by
25 sonication, and then insoluble material is removed by centrifugation. The MBP-ZFP proteins are captured on an amylose-bound resin, washed extensively with buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM DTT and 50 μ M ZnCl₂, then eluted with maltose in essentially the same buffer (purification is based on a standard protocol from NEB). Purified proteins are quantitated and stored for biochemical
30 analysis.

0007.20-0001660
TOP SECRET//NOFORN

The biochemical properties of the purified proteins, e.g., K_d , can be characterized by any suitable assay. In one embodiment, K_d is characterized via electrophoretic mobility shift assays (“EMSA”) (Buratowski & Chodosh, *in Current Protocols in Molecular Biology* pp. 12.2.1-12.2.7 (Ausubel ed., 1996); *see also* U.S. 5 Patent No. 5,789,538, co-owned PCT WO 00/42219, herein incorporated by reference). Affinity is measured by titrating purified protein against a low fixed amount of labeled double-stranded oligonucleotide target. The target comprises the natural binding site sequence (9 or 18 bp) flanked by the 3 bp found in the natural sequence. External to the binding site plus flanking sequence is a constant sequence. The annealed oligonucleotide 10 targets possess a 1 bp 5' overhang which allows for efficient labeling of the target with T4 phage polynucleotide kinase. For the assay the target is added at a concentration of 40 nM or lower (the actual concentration is kept at least 10-fold lower than the lowest protein dilution) and the reaction is allowed to equilibrate for at least 45 min. In addition the reaction mixture also contains 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM MgCl₂, 0.1 15 mM ZnCl₂, 5 mM DTT, 10% glycerol, 0.02% BSA (poly (dIdC) or (dAdT) (Pharmacia) can also added at 10-100 μ g/ μ l).

The equilibrated reactions are loaded onto a 10% polyacrylamide gel, which has been pre-run for 45 min in Tris/glycine buffer. Bound and unbound labeled target is resolved with electrophoresis at 150 V (alternatively, 10-20% gradient Tris-HCl 20 gels, containing a 4% polyacrylamide stacker, can be used). The dried gels are visualized by autoradiography or phosphoroimaging and the apparent K_d is determined by calculating the protein concentration that gives half-maximal binding.

Similar assays can also include determining active fractions in the protein preparations. Active fractions are determined by stoichiometric gel shifts where proteins 25 are titrated against a high concentration of target DNA. Titrations are done at 100, 50, and 25% of target (usually at micromolar levels).

In another embodiment, phage display libraries can be used to select zinc finger proteins with high affinity to the selected target site. This method differs fundamentally from direct design in that it involves the generation of diverse libraries of 30 mutagenized zinc finger proteins, followed by the isolation of proteins with desired DNA-

PCT/US00/07860

binding properties using affinity selection methods. To use this method, the experimenter typically proceeds as follows.

First, a gene for a zinc finger protein is mutagenized to introduce diversity into regions important for binding specificity and/or affinity. In a typical application, this 5 is accomplished via randomization of a single finger at positions -1, +2, +3, and +6, and perhaps accessory positions such as +1, +5, +8, or +10.

Next, the mutagenized gene is cloned into a phage or phagemid vector as a fusion with, e.g., gene III of filamentous phage, which encodes the coat protein pIII. The zinc finger gene is inserted between segments of gene III encoding the membrane export 10 signal peptide and the remainder of pIII, so that the zinc finger protein is expressed as an amino-terminal fusion with pIII in the mature, processed protein. When using phagemid vectors, the mutagenized zinc finger gene may also be fused to a truncated version of gene III encoding, minimally, the C-terminal region required for assembly of pIII into the phage particle.

15 The resultant vector library is transformed into *E. coli* and used to produce filamentous phage which express variant zinc finger proteins on their surface as fusions with the coat protein pIII (if a phagemid vector is used, then this step requires superinfection with helper phage). The phage library is then incubated with target DNA site, and affinity selection methods are used to isolate phage which bind target with high 20 affinity from bulk phage. Typically, the DNA target is immobilized on a solid support, which is then washed under conditions sufficient to remove all but the tightest binding phage. After washing, any phage remaining on the support are recovered via elution under conditions which totally disrupt zinc finger-DNA binding.

Recovered phage are used to infect fresh *E. coli*, which is then amplified 25 and used to produce a new batch of phage particles. The binding and recovery steps are then repeated as many times as is necessary to sufficiently enrich the phage pool for tight binders such that these may be identified using sequencing and/or screening methods.

Functional domains

30 A DNA-binding domain (e.g., a zinc finger domain) can optionally be associated with one or more regulatory domains for modulation of gene expression. The

10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800 810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960 970 980 990 1000

zinc finger protein can be covalently or non-covalently associated with one or more regulatory domains, alternatively two or more regulatory domains, with the two or more domains being two copies of the same domain, or two different domains. The regulatory domains can be covalently linked to the zinc finger protein, e.g., via an amino acid linker,
5 as part of a fusion protein. The zinc finger proteins can also be associated with a regulatory domain via a non-covalent dimerization domain, e.g., a leucine zipper, a STAT protein N terminal domain, or an FK506 binding protein (see, e.g., O'Shea, *Science* 254: 539 (1991), Barahmand-Pour *et al.*, *Curr. Top. Microbiol. Immunol.* 211:121-128 (1996); Klemm *et al.*, *Annu. Rev. Immunol.* 16:569-592 (1998); Klemm *et al.*, *Annu. Rev. Immunol.* 16:569-592 (1998); Ho *et al.*, *Nature* 382:822-826 (1996); and Pomeranz *et al.*, *Biochem.* 37:965 (1998)). The regulatory domain can be associated with the zinc finger protein at any suitable position, including the C- or N-terminus of the zinc finger protein.
10

Common regulatory domains for addition to the zinc finger protein include, e.g., effector domains from transcription factors (activators, repressors, co-activators, co-repressors), silencers, nuclear hormone receptors, oncogene transcription factors (e.g., myc, jun, fos, myb, max, mad, rel, ets, bcl, myb, mos family members etc.); DNA repair enzymes and their associated factors and modifiers; DNA rearrangement enzymes and their associated factors and modifiers; chromatin associated proteins and their modifiers (e.g., kinases, acetylases and deacetylases); and DNA modifying enzymes (e.g., methyltransferases, topoisomerases, helicases, ligases, kinases, phosphatases, polymerases, endonucleases) and their associated factors and modifiers.
15
20

Transcription factor polypeptides from which one can obtain a regulatory domain include those that are involved in regulated and basal transcription. Such polypeptides include transcription factors, their effector domains, coactivators, silencers, nuclear hormone receptors (see, e.g., Goodrich *et al.*, *Cell* 84:825-30 (1996) for a review of proteins and nucleic acid elements involved in transcription; transcription factors in general are reviewed in Barnes & Adcock, *Clin. Exp. Allergy* 25 Suppl. 2:46-9 (1995) and Roeder, *Methods Enzymol.* 273:165-71 (1996)). Databases dedicated to transcription factors are known (see, e.g., *Science* 269:630 (1995)). Nuclear hormone receptor transcription factors are described in, for example, Rosen *et al.*, *J. Med. Chem.* 38:4855-74 (1995). The C/EBP family of transcription factors are reviewed in Wedel *et al.*,
25
30

Immunobiology 193:171-85 (1995). Coactivators and co-repressors that mediate transcription regulation by nuclear hormone receptors are reviewed in, for example, Meier, *Eur. J. Endocrinol.* 134(2):158-9 (1996); Kaiser *et al.*, *Trends Biochem. Sci.* 21:342-5 (1996); and Utley *et al.*, *Nature* 394:498-502 (1998)). GATA transcription factors, which are involved in regulation of hematopoiesis, are described in, for example, Simon, *Nat. Genet.* 11:9-11 (1995); Weiss *et al.*, *Exp. Hematol.* 23:99-107. TATA box binding protein (TBP) and its associated TAF polypeptides (which include TAF30, TAF55, TAF80, TAF110, TAF150, and TAF250) are described in Goodrich & Tjian, *Curr. Opin. Cell Biol.* 6:403-9 (1994) and Hurley, *Curr. Opin. Struct. Biol.* 6:69-75 (1996). The STAT family of transcription factors are reviewed in, for example, Barahmand-Pour *et al.*, *Curr. Top. Microbiol. Immunol.* 211:121-8 (1996). Transcription factors involved in disease are reviewed in Aso *et al.*, *J. Clin. Invest.* 97:1561-9 (1996).

In one embodiment, the KRAB repression domain from the human KOX-1 protein is used as a transcriptional repressor (Thiesen *et al.*, *New Biologist* 2:363-374 (1990); Margolin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 91:4509-4513 (1994); Pengue *et al.*, *Nucl. Acids Res.* 22:2908-2914 (1994); Witzgall *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 91:4514-4518 (1994)). In another embodiment, KAP-1, a KRAB co-repressor, is used with KRAB (Friedman *et al.*, *Genes Dev.* 10:2067-2078 (1996)). Alternatively, KAP-1 can be used alone with a zinc finger protein. Other preferred transcription factors and transcription factor domains that act as transcriptional repressors include MAD (see, e.g., Sommer *et al.*, *J. Biol. Chem.* 273:6632-6642 (1998); Gupta *et al.*, *Oncogene* 16:1149-1159 (1998); Queva *et al.*, *Oncogene* 16:967-977 (1998); Larsson *et al.*, *Oncogene* 15:737-748 (1997); Laherty *et al.*, *Cell* 89:349-356 (1997); and Cultraro *et al.*, *Mol. Cell. Biol.* 17:2353-2359 (1997)); FKHR (forkhead in rhabdomyosarcoma gene; Ginsberg *et al.*, *Cancer Res.* 15:3542-3546 (1998); Epstein *et al.*, *Mol. Cell. Biol.* 18:4118-4130 (1998)); EGR-1 (early growth response gene product-1; Yan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:8298-8303 (1998); and Liu *et al.*, *Cancer Gene Ther.* 5:3-28 (1998)); the ets2 repressor factor repressor domain (ERD; Sgouras *et al.*, *EMBO J.* 14:4781-4793 (1995)); and the MAD smSIN3 interaction domain (SID; Ayer *et al.*, *Mol. Cell. Biol.* 16:5772-5781 (1996)).

In one embodiment, the HSV VP16 activation domain is used as a transcriptional activator (see, e.g., Hagmann *et al.*, *J. Virol.* 71:5952-5962 (1997)). Other preferred transcription factors that could supply activation domains include the VP64 activation domain (Seipel *et al.*, *EMBO J.* 11:4961-4968 (1996)); nuclear hormone receptors (see, e.g., Torchia *et al.*, *Curr. Opin. Cell. Biol.* 10:373-383 (1998)); the p65 subunit of nuclear factor kappa B (Bitko & Barik, *J. Virol.* 72:5610-5618 (1998) and Doyle & Hunt, *Neuroreport* 8:2937-2942 (1997)); and EGR-1 (early growth response gene product-1; Yan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:8298-8303 (1998); and Liu *et al.*, *Cancer Gene Ther.* 5:3-28 (1998)).

10 Kinases, phosphatases, and other proteins that modify polypeptides involved in gene regulation are also useful as regulatory domains for zinc finger proteins. Such modifiers are often involved in switching on or off transcription mediated by, for example, hormones. Kinases involved in transcription regulation are reviewed in Davis, *Mol. Reprod. Dev.* 42:459-67 (1995), Jackson *et al.*, *Adv. Second Messenger Phosphoprotein Res.* 28:279-86 (1993), and Boulikas, *Crit. Rev. Eukaryot. Gene Expr.* 5:1-77 (1995), while phosphatases are reviewed in, for example, Schonthal & Semin, *Cancer Biol.* 6:239-48 (1995). Nuclear tyrosine kinases are described in Wang, *Trends Biochem. Sci.* 19:373-6 (1994).

20 As described, useful domains can also be obtained from the gene products of oncogenes (e.g., myc, jun, fos, myb, max, mad, rel, ets, bcl, myb, mos family members) and their associated factors and modifiers. Oncogenes are described in, for example, Cooper, *Oncogenes, The Jones and Bartlett Series in Biology* (2nd ed., 1995). The ets transcription factors are reviewed in Waslylk *et al.*, *Eur. J. Biochem.* 211:7-18 (1993) and Crepieux *et al.*, *Crit. Rev. Oncog.* 5:615-38 (1994). Myc oncogenes are reviewed in, for example, Ryan *et al.*, *Biochem. J.* 314:713-21 (1996). The jun and fos transcription factors are described in, for example, *The Fos and Jun Families of Transcription Factors* (Angel & Herrlich, eds. 1994). The max oncogene is reviewed in Hurlin *et al.*, *Cold Spring Harb. Symp. Quant. Biol.* 59:109-16. The myb gene family is reviewed in Kanei-Ishii *et al.*, *Curr. Top. Microbiol. Immunol.* 211:89-98 (1996). The mos family is reviewed in Yew *et al.*, *Curr. Opin. Genet. Dev.* 3:19-25 (1993).

Zinc finger proteins can include regulatory domains obtained from DNA repair enzymes and their associated factors and modifiers. DNA repair systems are reviewed in, for example, Vos, *Curr. Opin. Cell Biol.* 4:385-95 (1992); Sancar, *Ann. Rev. Genet.* 29:69-105 (1995); Lehmann, *Genet. Eng.* 17:1-19 (1995); and Wood, *Ann. Rev. Biochem.* 65:135-67 (1996). DNA rearrangement enzymes and their associated factors and modifiers can also be used as regulatory domains (see, e.g., Gangloff *et al.*, *Experientia* 50:261-9 (1994); Sadowski, *FASEB J.* 7:760-7 (1993)).

Similarly, regulatory domains can be derived from DNA modifying enzymes (e.g., DNA methyltransferases, topoisomerases, helicases, ligases, kinases, phosphatases, polymerases) and their associated factors and modifiers. Helicases are reviewed in Matson *et al.*, *Bioessays*, 16:13-22 (1994), and methyltransferases are described in Cheng, *Curr. Opin. Struct. Biol.* 5:4-10 (1995). Chromatin associated proteins and their modifiers (e.g., kinases, acetylases and deacetylases), such as histone deacetylase (Wolffe, *Science* 272:371-2 (1996)) are also useful as domains for addition to the zinc finger protein of choice. In one preferred embodiment, the regulatory domain is a DNA methyl transferase that acts as a transcriptional repressor (see, e.g., Van den Wyngaert *et al.*, *FEBS Lett.* 426:283-289 (1998); Flynn *et al.*, *J. Mol. Biol.* 279:101-116 (1998); Okano *et al.*, *Nucleic Acids Res.* 26:2536-2540 (1998); and Zardo & Caiafa, *J. Biol. Chem.* 273:16517-16520 (1998)). In another preferred embodiment, endonucleases such as Fok1 are used as transcriptional repressors, which act via gene cleavage (see, e.g., WO95/09233; and PCT/US94/01201).

Factors that control chromatin and DNA structure, movement and localization and their associated factors and modifiers; factors derived from microbes (e.g., prokaryotes, eukaryotes and virus) and factors that associate with or modify them can also be used to obtain chimeric proteins. In one embodiment, recombinases and integrases are used as regulatory domains. In one embodiment, histone acetyltransferase is used as a transcriptional activator (see, e.g., Jin & Scotto, *Mol. Cell. Biol.* 18:4377-4384 (1998); Wolffe, *Science* 272:371-372 (1996); Taunton *et al.*, *Science* 272:408-411 (1996); and Hassig *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95:3519-3524 (1998)). In another embodiment, histone deacetylase is used as a transcriptional repressor (see, e.g., Jin & Scotto, *Mol. Cell. Biol.* 18:4377-4384 (1998); Syntichaki & Thireos, *J. Biol. Chem.*

273:24414-24419 (1998); Sakaguchi *et al.*, *Genes Dev.* 12:2831-2841 (1998); and Martinez *et al.*, *J. Biol. Chem.* 273:23781-23785 (1998)).

Another suitable repression domain is methyl binding domain protein 2B (MBD-2B) (see, also Hendrich et al. (1999) *Mamm Genome* 10:906-912 for description of MBD proteins). Another useful repression domain is that associated with the v-ErbA protein (see *infra*). See, for example, Damm, et al. (1989) *Nature* 339:593-597; Evans (1989) *Int. J. Cancer Suppl.* 4:26-28; Pain et al. (1990) *New Biol.* 2:284-294; Sap et al. (1989) *Nature* 340:242-244; Zenke et al. (1988) *Cell* 52:107-119; and Zenke et al. (1990) *Cell* 61:1035-1049. Additional exemplary repression domains include, but are not limited to, thyroid hormone receptor (TR, see *infra*), SID, MBD1, MBD2, MBD3, MBD4, MBD-like proteins, members of the DNMT family (e.g., DNMT1, DNMT3A, DNMT3B), Rb, MeCP1 and MeCP2. See, for example, Bird et al. (1999) *Cell* 99:451-454; Tyler et al. (1999) *Cell* 99:443-446; Knoepfler et al. (1999) *Cell* 99:447-450; and Robertson et al. (2000) *Nature Genet.* 25:338-342. Additional exemplary repression domains include, but are not limited to, ROM2 and AtHD2A. See, for example, Chern et al. (1996) *Plant Cell* 8:305-321; and Wu et al. (2000) *Plant J.* 22:19-27.

Certain members of the nuclear hormone receptor (NHR) superfamily, including, for example, thyroid hormone receptors (TRs) and retinoic acid receptors (RARs) are among the most potent transcriptional regulators currently known. Zhang *et al.*, *Annu. Rev. Physiol.* 62:439-466 (2000) and Sucov *et al.*, *Mol Neurobiol* 10(2-3):169-184 (1995). In the absence of their cognate ligand, these proteins bind with high specificity and affinity to short stretches of DNA (e.g., 12-17 base pairs) within regulatory loci (e.g., enhancers and promoters) and effect robust transcriptional repression of adjacent genes. The potency of their regulatory action stems from the concurrent use of two distinct functional pathways to drive gene silencing: (i) the creation of a localized domain of repressive chromatin *via* the targeting of a complex between the corepressor N-CoR and a histone deacetylase, HDAC3 (Guenther *et al.*, *Genes Dev* 14:1048-1057 (2000); Urnov *et al.*, *EMBO J* 19:4074-4090 (2000); Li *et al.*, *EMBO J* 19, 4342-4350 (2000) and Underhill *et al.*, *J. Biol. Chem.* 275:40463-40470 (2000)) and (ii) a chromatin-independent pathway (Urnov *et al.*, *supra*) that may involve direct interference

with the function of the basal transcription machinery (Fondell *et al.*, *Genes Dev* 7(7B):1400-1410 (1993) and Fondell *et al.*, *Mol Cell Biol* 16:281-287 (1996)).

In the presence of very low (*e.g.*, nanomolar) concentrations of their ligand, these receptors undergo a conformational change which leads to the release of 5 corepressors, recruitment of a different class of auxiliary molecules (*e.g.*, coactivators) and potent transcriptional activation. Collingwood *et al.*, *J. Mol. Endocrinol.* 23(3):255-275 (1999).

The portion of the receptor protein responsible for transcriptional control (10 *e.g.*, repression and activation) can be physically separated from the portion responsible for DNA binding, and retains full functionality when tethered to other polypeptides, for example, other DNA-binding domains. Accordingly, a nuclear hormone receptor transcription control domain can be fused to a ZFP DNA-binding domain such that the transcriptional regulatory activity of the receptor can be targeted to a chromosomal region of interest (*e.g.*, a gene) by virtue of the ZFP binding domain.

Moreover, the structure of TR and other nuclear hormone receptors can be altered, either naturally or through recombinant techniques, such that it loses all capacity to respond to hormone (thus losing its ability to drive transcriptional activation), but retains the ability to effect transcriptional repression. This approach is exemplified by the transcriptional regulatory properties of the oncoprotein v-ErbA. The v-ErbA protein is 15 one of the two proteins required for leukemic transformation of immature red blood cell precursors in young chicks by the avian erythroblastosis virus. TR is a major regulator of erythropoiesis (Beug *et al.*, *Biochim Biophys Acta* 1288(3):M35-47 (1996); in particular, in its unliganded state, it represses genes required for cell cycle arrest and the differentiated state. Thus, the administration of thyroid hormone to immature 20 erythroblasts leads to their rapid differentiation. The v-ErbA oncoprotein is an extensively mutated version of TR; these mutations include: (i) deletion of 12 amino-terminal amino acids; (ii) fusion to the *gag* oncoprotein; (iii) several point mutations in the DNA binding domain that alter the DNA binding specificity of the protein relative to its parent, TR, and impair its ability to heterodimerize with the retinoid X receptor; (iv) 25 multiple point mutations in the ligand-binding domain of the protein that effectively eliminate the capacity to bind thyroid hormone; and (v) a deletion of a carboxy-terminal

0 9 9 1 5 0 6 2 6 0

stretch of amino acids that is essential for transcriptional activation. Stunnenberg *et al.*, *Biochim Biophys Acta* 1423(1):F15-33 (1999). As a consequence of these mutations, v-ErbA retains the capacity to bind to naturally occurring TR target genes and is an effective transcriptional repressor when bound (Urnov *et al.*, *supra*; Sap *et al.*, *Nature* 5 340:242-244 (1989); and Ciana *et al.*, *EMBO J.* 17(24):7382-7394 (1999). In contrast to TR, however, v-ErbA is completely insensitive to thyroid hormone, and thus maintains transcriptional repression in the face of a challenge from any concentration of thyroids or retinoids, whether endogenous to the medium, or added by the investigator (4).

We have shown that this functional property of v-ErbA is retained when its 10 repression domain is fused to a heterologous, synthetic DNA binding domain. Accordingly, in one aspect, v-ErbA or its functional fragments are used as a repression domain. In additional embodiments, TR or its functional domains are used as a 15 repression domain in the absence of ligand and/or as an activation domain in the presence of ligand (e.g., 3,5,3'-triiodo-L-thyronine or T3). Thus, TR can be used as a switchable functional domain (*i.e.*, a bifunctional domain); its activity (activation or repression) 20 being dependent upon the presence or absence (respectively) of ligand.

Additional exemplary repression domains are obtained from the DAX protein and its functional fragments. Zazopoulos *et al.*, *Nature* 390:311-315 (1997). In particular, the C-terminal portion of DAX-1, including amino acids 245-470, has been 25 shown to possess repression activity. Altincicek *et al.*, *J. Biol. Chem.* 275:7662-7667 (2000). A further exemplary repression domain is the RBP1 protein and its functional fragments. Lai *et al.*, *Oncogene* 18:2091-2100 (1999); Lai *et al.*, *Mol. Cell. Biol.* 19:6632-6641 (1999); Lai *et al.*, *Mol. Cell. Biol.* 21:2918-2932 (2001) and WO 01/04296. The full-length RBP1 polypeptide contains 1257 amino acids. Exemplary functional fragments of RBP1 are a polypeptide comprising amino acids 1114-1257, and 30 a polypeptide comprising amino acids 243-452.

Members of the TIEG family of transcription factors contain three 35 repression domains known as R1, R2 and R3. Repression by TIEG family proteins is achieved at least in part through recruitment of mSIN3A histone deacetylases complexes. Cook *et al.* (1999) *J. Biol. Chem.* 274:29,500-29,504; Zhang *et al.* (2001) *Mol. Cell. Biol.* 21:5041-5049. Any or all of these repression domains (or their functional

TO BE DELETED

fragments) can be fused alone, or in combination with additional repression domains (or their functional fragments), to a DNA-binding domain to generate a targeted exogenous repressor molecule.

Furthermore, the product of the human cytomegalovirus (HCMV) UL34 5 open reading frame acts as a transcriptional repressor of certain HCMV genes, for example, the US3 gene. LaPierre *et al.* (2001) *J. Virol.* **75**:6062-6069. Accordingly, the UL34 gene product, or functional fragments thereof, can be used as a component of a fusion polypeptide also comprising a zinc finger binding domain. Nucleic acids encoding such fusions are also useful in the methods and compositions disclosed herein.

10 Yet another exemplary repression domain is the CDF-1 transcription factor and/or its functional fragments. *See*, for example, WO 99/27092.

15 The Ikaros family of proteins are involved in the regulation of lymphocyte development, at least in part by transcriptional repression. Accordingly, an Ikaros family member (*e.g.*, Ikaros, Aiolos) or a functional fragment thereof, can be used as a repression domain. *See*, for example, Sabbattini *et al.* (2001) *EMBO J.* **20**:2812-2822.

20 The yeast Ash1p protein comprises a transcriptional repression domain. Maxon *et al.* (2001) *Proc. Natl. Acad. Sci. USA* **98**:1495-1500. Accordingly, the Ash1p protein, its functional fragments, and homologues of Ash1p, such as those found, for example, in, vertebrate, mammalian, and plant cells, can serve as a repression domain for use in the methods and compositions disclosed herein.

25 Additional exemplary repression domains include those derived from histone deacetylases (HDACs, *e.g.*, Class I HDACs, Class II HDACs, SIR-2 homologues), HDAC-interacting proteins (*e.g.*, SIN3, SAP30, SAP15, NCoR, SMRT, RB, p107, p130, RBAP46/48, MTA, Mi-2, Brg1, Brm), DNA-cytosine methyltransferases (*e.g.*, Dnmt1, Dnmt3a, Dnmt3b), proteins that bind methylated DNA (*e.g.*, MBD1, MBD2, MBD3, MBD4, MeCP2, DMAP1), protein methyltransferases (*e.g.*, lysine and arginine methylases, SuVar homologues such as Suv39H1), polycomb-type repressors (*e.g.*, Bmi-1, eed1, RING1, RYBP, E2F6, Mel18, YY1 and CtBP), viral repressors (*e.g.*, adenovirus E1b 55K protein, cytomegalovirus UL34 protein, viral oncogenes such as v-erbA), hormone receptors (*e.g.*, Dax-1, estrogen receptor, thyroid hormone receptor), and repression domains associated with naturally-occurring zinc

T002800 05474560

finger proteins (e.g., WT1, KAP1). Further exemplary repression domains include members of the polycomb complex and their homologues, HPH1, HPH2, HPC2, NC2, groucho, Eve, tramtrak, mHP1, SIP1, ZEB1, ZEB2, and Enx1/Ezh2. In all of these cases, either the full-length protein or a functional fragment can be used as a repression domain
5 for fusion to a zinc finger binding domain. Furthermore, any homologues of the aforementioned proteins can also be used as repression domains, as can proteins (or their functional fragments) that interact with any of the aforementioned proteins.

Additional repression domains, and exemplary functional fragments, are as follows. Hes1 is a human homologue of the *Drosophila hairy* gene product and
10 comprises a functional fragment encompassing amino acids 910-1014. In particular, a WRPW (trp-arg-pro-trp) motif can act as a repression domain. Fisher *et al.* (1996) *Mol. Cell. Biol.* **16**:2670-2677.

The TLE1, TLE2 and TLE3 proteins are human homologues of the *Drosophila groucho* gene product. Functional fragments of these proteins possessing
15 repression activity reside between amino acids 1-400. Fisher *et al.*, *supra*.

The Tbx3 protein possesses a functional repression domain between amino acids 524-721. He *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96**:10,212-10,217. The Tbx2 gene product is involved in repression of the p14/p16 genes and contains a region
20 between amino acids 504-702 that is homologous to the repression domain of Tbx3; accordingly Tbx2 and/or this functional fragment can be used as a repression domain. Carreira *et al.* (1998) *Mol. Cell. Biol.* **18**:5,099-5,108.

The human Ezh2 protein is a homologue of *Drosophila enhancer of zeste* and recruits the eed1 *polycomb*-type repressor. A region of the Ezh2 protein comprising
25 amino acids 1-193 can interact with eed1 and repress transcription; accordingly Ezh2 and/or this functional fragment can be used as a repression domain. Denisenko *et al.* (1998) *Mol. Cell. Biol.* **18**:5634-5642.

The RYBP protein is a corepressor that interacts with *polycomb* complex members and with the YY1 transcription factor. A region of RYBP comprising amino acids 42-208 has been identified as functional repression domain. Garcia *et al.* (1999)
30 *EMBO J.* **18**:3404-3418.

TELEGRAMS - DRAFTS - PROOFS -

The RING finger protein RING1A is a member of two different vertebrate *polycomb*-type complexes, contains multiple binding sites for various components of the *polycomb* complex, and possesses transcriptional repression activity. Accordingly, RING1A or its functional fragments can serve as a repression domain. Satjin *et al.*

5 (1997) *Mol. Cell. Biol.* 17:4105-4113.

The Bmi-1 protein is a member of a vertebrate *polycomb* complex and is involved in transcriptional silencing. It contains multiple binding sites for various *polycomb* complex components. Accordingly, Bmi-1 and its functional fragments are useful as repression domains. Gunster *et al.* (1997) *Mol. Cell. Biol.* 17:2326-2335;

10 Hemenway *et al.* (1998) *Oncogene* **16**:2541-2547.

The E2F6 protein is a member of the mammalian Bmi-1-containing *polycomb* complex and is a transcriptional repressor that is capable of recruiting RYBP, Bmi-1 and RING1A. A functional fragment of E2F6 comprising amino acids 129-281 acts as a transcriptional repression domain. Accordingly, E2F6 and its functional fragments can be used as repression domains. Trimarchi *et al.* (2001) *Proc Natl. Acad. Sci. USA* **98**:1519-1524.

The eed1 protein represses transcription at least in part through recruitment of histone deacetylases (e.g., HDAC2). Repression activity resides in both the N- and C-terminal regions of the protein. Accordingly, eed1 and its functional fragments can be used as repression domains. van der Vlag *et al.* (1999) *Nature Genet.* **23**:474-478.

The CTBP2 protein represses transcription at least in part through recruitment of an HPC2-*polycomb* complex. Accordingly, CTBP2 and its functional fragments are useful as repression domains. Richard *et al.* (1999) *Mol. Cell. Biol.* **19**:777-787.

25 Neuron-restrictive silencer factors are proteins that repress expression of neuron-specific genes. Accordingly, a NRSF or functional fragment thereof can serve as a repression domain. *See, for example, US Patent No. 6,270,990.*

It will be clear to those of skill in the art that, in the formation of a fusion protein (or a nucleic acid encoding same) between a zinc finger binding domain and a functional domain, either a repressor or a molecule that interacts with a repressor is suitable as a functional domain. Essentially any molecule capable of recruiting a

repressive complex and/or repressive activity (such as, for example, histone deacetylation) to the target gene is useful as a repression domain of a fusion protein.

Additional exemplary activation domains include, but are not limited to, p300, CBP, PCAF, SRC1 PvALF, AtHD2A and ERF-2. See, for example, Robyr et al. 5 (2000) Mol. Endocrinol. 14:329-347; Collingwood et al. (1999) J. Mol. Endocrinol. 23:255-275; Leo et al. (2000) Gene 245:1-11; Manteuffel-Cymborowska (1999) Acta Biochim. Pol. 46:77-89; McKenna et al. (1999) J. Steroid Biochem. Mol. Biol. 69:3-12; Malik et al. (2000) Trends Biochem. Sci. 25:277-283; and Lemon et al. (1999) Curr. 10 Opin. Genet. Dev. 9:499-504. Additional exemplary activation domains include, but are not limited to, OsGAI, HALF-1, C1, AP1, ARF-5, -6, -7, and -8, CPRF1, CPRF4, MYC-RP/GP, and TRAB1. See, for example, Ogawa et al. (2000) Gene 245:21-29; Okanami et al. (1996) Genes Cells 1:87-99; Goff et al. (1991) Genes Dev. 5:298-309; Cho et al. 15 (1999) Plant Mol. Biol. 40:419-429; Ulmason et al. (1999) Proc. Natl. Acad. Sci. USA 96:5844-5849; Sprenger-Haussels et al. (2000) Plant J. 22:1-8; Gong et al. (1999) Plant Mol. Biol. 41:33-44; and Hobo et al. (1999) Proc. Natl. Acad. Sci. USA 96:15,348-15,353.

It will be clear to those of skill in the art that, in the formation of a fusion protein (or a nucleic acid encoding same) between a zinc finger binding domain and a functional domain, either an activator or a molecule that interacts with an activator is 20 suitable as a functional domain. Essentially any molecule capable of recruiting an activating complex and/or activating activity (such as, for example, histone acetylation) to the target gene is useful as an activating domain of a fusion protein.

Insulator domains, chromatin remodeling proteins such as ISWI-containing domains and/or methyl binding domain proteins suitable for use as functional 25 domains in fusion molecules are described, for example, in co-owned PCT application US01/40616 and co-owned U.S. Patent applications 60/236,409; 60/236,884; and 60/253,678.

In a further embodiment, a DNA-binding domain (e.g., a zinc finger domain) is fused to a bifunctional domain (BFD). A bifunctional domain is a 30 transcriptional regulatory domain whose activity depends upon interaction of the BFD with a second molecule. The second molecule can be any type of molecule capable of

US01/40616

influencing the functional properties of the BFD including, but not limited to, a compound, a small molecule, a peptide, a protein, a polysaccharide or a nucleic acid. An exemplary BFD is the ligand binding domain of the estrogen receptor (ER). In the presence of estradiol, the ER ligand binding domain acts as a transcriptional activator; 5 while, in the absence of estradiol and the presence of tamoxifen or 4-hydroxy-tamoxifen, it acts as a transcriptional repressor. Another example of a BFD is the thyroid hormone receptor (TR) ligand binding domain which, in the absence of ligand, acts as a transcriptional repressor and in the presence of thyroid hormone (T3), acts as a transcriptional activator. An additional BFD is the glucocorticoid receptor (GR) ligand 10 binding domain. In the presence of dexamethasone, this domain acts as a transcriptional activator; while, in the presence of RU486, it acts as a transcriptional repressor. An additional exemplary BFD is the ligand binding domain of the retinoic acid receptor. In the presence of its ligand all-trans-retinoic acid, the retinoic acid receptor recruits a number of co-activator complexes and activates transcription. In the absence of ligand, 15 the retinoic acid receptor is not capable of recruiting transcriptional co-activators. Additional BFDs are known to those of skill in the art. See, for example, US Patent Nos. 5,834,266 and 5,994,313 and PCT WO 99/10508.

20 Examples of the ability of various functional domains to regulate gene expression are provided in co-owned patent application entitled "Modulation of Endogenous Gene Expression in Cells," reference S2-US5, filed even date herewith, the disclosure of which is hereby incorporated by reference in its entirety.

Linker domains between polypeptide domains, e.g., between two zinc 25 finger proteins or between a zinc finger protein and a regulatory domain, can be included. Such linkers are typically polypeptide sequences, such as poly gly sequences of between about 5 and 200 amino acids. Preferred linkers are typically flexible amino acid subsequences which are synthesized as part of a recombinant fusion protein. For example, in one embodiment, the linker DGGGS (SEQ ID NO: 4) is used to link two zinc finger proteins. In another embodiment, the flexible linker linking two zinc finger proteins is an amino acid subsequence comprising the sequence TGEKP (SEQ ID NO: 5) 30 (see, e.g., Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 5525-5530 (1997)). In another embodiment, the linker LRQKDGERP (SEQ ID NO: 6) is used to link two zinc finger

proteins. In another embodiment, the following linkers are used to link two zinc finger proteins: GGRR (SEQ ID NO: 7) (Pomerantz *et al.* 1995, *supra*), (G₄S)_n (SEQ ID NO: 8) (Kim *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 93, 1156-1160 (1996.); and GGRRGGGS (SEQ ID NO: 9); LRQRDGERP (SEQ ID NO: 10); LRQKDGGGSERP (SEQ ID NO: 11);
5 LRQKD(G₃S)₂ERP (SEQ ID NO: 12). Alternatively, flexible linkers can be rationally designed using computer program capable of modeling both DNA-binding sites and the peptides themselves (Desjarlais & Berg, *Proc. Natl. Acad. Sci. U.S.A.* 90:2256-2260 (1993), *Proc. Natl. Acad. Sci. U.S.A.* 91:11099-11103 (1994) or by phage display methods.

10 In other embodiments, a chemical linker is used to connect synthetically or recombinantly produced domain sequences. Such flexible linkers are known to persons of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages. In addition to covalent
15 linkage of zinc finger proteins to regulatory domains, non-covalent methods can be used to produce molecules with zinc finger proteins associated with regulatory domains.

In addition to regulatory domains, often the zinc finger protein is expressed as a fusion protein such as maltose binding protein (“MBP”), glutathione S transferase (GST), hexahistidine, c-myc, and the FLAG epitope, for ease of purification, monitoring expression, or monitoring cellular and subcellular localization.
20

Subcloning and expression of nucleic acids encoding zinc finger protein

The nucleic acid encoding the zinc finger protein of choice is typically cloned into vectors for transformation into prokaryotic or eukaryotic cells for replication, expression, e.g., for determination of K_d. Such vectors are typically prokaryote vectors, e.g., plasmids, or shuttle vectors, or eukaryotic vectors such insect vectors, for storage or manipulation of the nucleic acid encoding zinc finger protein or production of protein, or eukaryotic vector such as viral vectors (e.g., adenoviral vectors, retroviral vector, etc.) for expression of zinc finger proteins and optionally regulation of gene expression. The
25 nucleic acid encoding a zinc finger protein can then be administered to a plant cell,
30

TOP SECRET - DEFENSE

animal cell, a mammalian cell or a human cell, a fungal cell, a bacterial cell, or a protozoal cell.

To obtain expression of a cloned gene or nucleic acid, a zinc finger protein is typically subcloned into an expression vector that contains a promoter to direct transcription. Suitable bacterial and eukaryotic promoters are well known in the art and described, e.g., in Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 5 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994). Bacterial expression systems for expressing the zinc finger protein are available in, e.g., *E. coli*, *Bacillus sp.*, 10 and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available.

The promoter used to direct expression of a zinc finger protein nucleic acid depends on the particular application. For example, a strong constitutive promoter is 15 typically used for expression and purification of zinc finger protein. In contrast, when a zinc finger protein is administered *in vivo* for gene regulation, either a constitutive or an inducible promoter is used, depending on the particular use of the zinc finger protein. The promoter typically can also include elements that are responsive to transactivation, e.g., hypoxia response elements, Gal4 response elements, lac repressor response element, 20 and small molecule control systems such as tet-regulated systems and the RU-486 system (see, e.g., Gossen & Bujard, *Proc. Natl. Acad. Sci. U.S.A.* 89:5547 (1992); Oligino *et al.*, *Gene Ther.* 5:491-496 (1998); Wang *et al.*, *Gene Ther.* 4:432-441 (1997); Neering *et al.*, *Blood* 88:1147-1155 (1996); and Rendahl *et al.*, *Nat. Biotechnol.* 16:757-761 (1998)).

In addition to the promoter, the expression vector typically contains a 25 transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells, either prokaryotic or eukaryotic. A typical expression cassette thus contains a promoter operably linked, e.g., to the nucleic acid sequence encoding the zinc finger protein, and signals required, e.g., for efficient polyadenylation of the transcript, transcriptional termination, ribosome binding sites, or 30 translation termination. Additional elements of the cassette may include, e.g., enhancers, and heterologous spliced intronic signals.

100-0007-20-00000

The particular expression vector used to transport the genetic information into the cell is selected with regard to the intended use of the zinc finger protein, e.g., expression in plants, animals, bacteria, fungus, protozoa etc. (see expression vectors described below and in the Example section). Standard bacterial expression vectors 5 include plasmids such as pBR322 based plasmids, pSKF, pET23D, and commercially available fusion expression systems such as GST and LacZ. A preferred fusion protein is the maltose binding protein, "MBP." Such fusion proteins are used for purification of the zinc finger protein. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, for monitoring expression, and for monitoring cellular 10 and subcellular localization, e.g., c-myc or FLAG.

Expression vectors containing regulatory elements from eukaryotic viruses are often used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, 15 and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 late promoter, CMV promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Some expression systems have markers for selection of stably transfected 20 cell lines such as neomycin, thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. High yield expression systems are also suitable, such as using a baculovirus vector in insect cells, with a zinc finger protein encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include 25 a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of recombinant sequences.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of protein, which are then purified 30 using standard techniques (see, e.g., Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification, in Methods in Enzymology*, vol. 182 (Deutscher,

ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983)).

Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, naked DNA, plasmid vectors, viral vectors, both episomal and integrative, and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (see, e.g., Sambrook *et al.*, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the protein of choice.

Vectors

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding engineered zinc finger protein in mammalian cells or target tissues. Such methods can be used to administer nucleic acids encoding zinc finger proteins to cells *in vitro* or *in vivo*. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) (1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994).

Methods of non-viral delivery of nucleic acids encoding engineered zinc finger proteins include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial

100-920-000560

virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., US 5,049,386, US 4,946,787; and US 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of 5 Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 10 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

The use of RNA or DNA viral based systems for the delivery of nucleic acids encoding engineered zinc finger protein take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to subjects (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of zinc finger proteins could include 15 retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Viral vectors are currently the most efficient and versatile method of gene transfer in target cells and tissues. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in 20 long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues. 25

The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vector that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would 30 therefore depend on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The

100-0000-0000-0000

minimum *cis*-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), simian immuno-deficiency virus (SIV), human immuno-deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher *et al.*, *J. Virol.* 66:2731-2739 (1992); Johann *et al.*, *J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al.*, *Virol.* 176:58-59 (1990); Wilson *et al.*, *J. Virol.* 63:2374-2378 (1989); Miller *et al.*, *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).

In applications where transient expression of the zinc finger protein is preferred, adenoviral based systems are typically used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, e.g., in the *in vitro* production of nucleic acids and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (see, e.g., West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *Proc. Natl. Acad. Sci. U.S.A.* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989).

Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the protein to be expressed. The missing viral functions are supplied in *trans* by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required

100-0007.20-0007.20

for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely *rep* and *cap*, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from 5 the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV.

In many situations, it is desirable that the vector be delivered with a high degree of specificity to a particular tissue type. A viral vector is typically modified to 10 have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the viruses outer surface. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant 15 virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other pairs of virus expressing a ligand fusion protein and target cell expressing a receptor. For example, filamentous phage can be engineered to display antibody fragments (e.g., FAB or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies 20 primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences thought to favor uptake by specific target cells.

Expression vectors can be delivered *in vivo* by administration to an individual subject, typically by systemic administration (e.g., intravenous, intraperitoneal, 25 intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, naked DNA can be administered. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual subject (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection 30 for cells which have incorporated the vector.

100-9200-0007.20

Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a 5 particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations 10 of pharmaceutical compositions available, as described below (see, e.g., *Remington's Pharmaceutical Sciences*, 17th ed., 1989).

Delivery vehicles

An important factor in the administration of polypeptide compounds, such 15 as the zinc finger proteins, is ensuring that the polypeptide has the ability to traverse the plasma membrane of a cell, or the membrane of an intra-cellular compartment such as the nucleus. Cellular membranes are composed of lipid-protein bilayers that are freely permeable to small, nonionic lipophilic compounds and are inherently impermeable to polar compounds, macromolecules, and therapeutic or diagnostic agents. However, 20 proteins and other compounds such as liposomes have been described, which have the ability to translocate polypeptides such as zinc finger proteins across a cell membrane.

For example, "membrane translocation polypeptides" have amphiphilic or 25 hydrophobic amino acid subsequences that have the ability to act as membrane-translocating carriers. In one embodiment, homeodomain proteins have the ability to translocate across cell membranes. The shortest internalizable peptide of a homeodomain protein, Antennapedia, was found to be the third helix of the protein, from amino acid position 43 to 58 (see, e.g., Prochiantz, *Current Opinion in Neurobiology* 6:629-634 (1996)). Another subsequence, the h (hydrophobic) domain of signal peptides, was found 30 to have similar cell membrane translocation characteristics (see, e.g., Lin *et al.*, *J. Biol. Chem.* 270:1 4255-14258 (1995)).

100-100-100-100-100-100-100-100-100-100

Examples of peptide sequences which can be linked to a protein, for facilitating uptake of the protein into cells, include, but are not limited to: an 11 amino acid peptide of the tat protein of HIV; a 20 residue peptide sequence which corresponds to amino acids 84-103 of the p16 protein (see Fahraeus *et al.*, *Current Biology* 6:84 (1996)); the third helix of the 60-amino acid long homeodomain of Antennapedia (Derossi *et al.*, *J. Biol. Chem.* 269:10444 (1994)); the h region of a signal peptide such as the Kaposi fibroblast growth factor (K-FGF) h region (Lin *et al.*, *supra*); or the VP22 translocation domain from HSV (Elliot & O'Hare, *Cell* 88:223-233 (1997)). Other suitable chemical moieties that provide enhanced cellular uptake may also be chemically linked to zinc finger proteins.

Toxin molecules also have the ability to transport polypeptides across cell membranes. Often, such molecules are composed of at least two parts (called "binary toxins"): a translocation or binding domain or polypeptide and a separate toxin domain or polypeptide. Typically, the translocation domain or polypeptide binds to a cellular receptor, and then the toxin is transported into the cell. Several bacterial toxins, including *Clostridium perfringens* iota toxin, diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE), pertussis toxin (PT), *Bacillus anthracis* toxin, and pertussis adenylate cyclase (CYA), have been used in attempts to deliver peptides to the cell cytosol as internal or amino-terminal fusions (Arora *et al.*, *J. Biol. Chem.*, 268:3334-3341 (1993); Perelle *et al.*, *Infect. Immun.*, 61:5147-5156 (1993); Stenmark *et al.*, *J. Cell Biol.* 113:1025-1032 (1991); Donnelly *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:3530-3534 (1993); Carbonetti *et al.*, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 95:295 (1995); Sebo *et al.*, *Infect. Immun.* 63:3851-3857 (1995); Klimpel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:10277-10281 (1992); and Novak *et al.*, *J. Biol. Chem.* 267:17186-17193 (1992)).

Such subsequences can be used to translocate zinc finger proteins across a cell membrane. zinc finger proteins can be conveniently fused to or derivatized with such sequences. Typically, the translocation sequence is provided as part of a fusion protein. Optionally, a linker can be used to link the zinc finger protein and the translocation sequence. Any suitable linker can be used, e.g., a peptide linker.

The zinc finger protein can also be introduced into an animal cell, preferably a mammalian cell, via a liposomes and liposome derivatives such as

2025 2020 2015 2010 2005 2000 1995 1990 1985 1980 1975 1970 1965 1960

immunoliposomes. The term "liposome" refers to vesicles comprised of one or more concentrically ordered lipid bilayers, which encapsulate an aqueous phase. The aqueous phase typically contains the compound to be delivered to the cell, i.e., a zinc finger protein.

5 The liposome fuses with the plasma membrane, thereby releasing the drug into the cytosol. Alternatively, the liposome is phagocytosed or taken up by the cell in a transport vesicle. Once in the endosome or phagosome, the liposome either degrades or fuses with the membrane of the transport vesicle and releases its contents.

10 In current methods of drug delivery via liposomes, the liposome ultimately becomes permeable and releases the encapsulated compound (in this case, a zinc finger protein) at the target tissue or cell. For systemic or tissue specific delivery, this can be accomplished, for example, in a passive manner wherein the liposome bilayer degrades over time through the action of various agents in the body. Alternatively, active drug release involves using an agent to induce a permeability change in the liposome vesicle.

15 Liposome membranes can be constructed so that they become destabilized when the environment becomes acidic near the liposome membrane (*see, e.g., Proc. Natl. Acad. Sci. U.S.A.* 84:7851 (1987); *Biochemistry* 28:908 (1989)). When liposomes are endocytosed by a target cell, for example, they become destabilized and release their contents. This destabilization is termed fusogenesis. Dioleoylphosphatidylethanolamine

20 (DOPE) is the basis of many "fusogenic" systems.

Such liposomes typically comprise a zinc finger protein and a lipid component, e.g., a neutral and/or cationic lipid, optionally including a receptor-recognition molecule such as an antibody that binds to a predetermined cell surface receptor or ligand (e.g., an antigen). A variety of methods are available for preparing liposomes as described in, e.g., Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,946,787, PCT Publication No. WO 91\17424, Deamer & Bangham, *Biochim. Biophys. Acta* 443:629-634 (1976); Fraley, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 76:3348-3352 (1979); Hope *et al.*, *Biochim. Biophys. Acta* 812:55-65 (1985); Mayer *et al.*, *Biochim. Biophys. Acta* 858:161-168 (1986); Williams *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 85:242-

246 (1988); *Liposomes* (Ostro (ed.), 1983, Chapter 1); Hope *et al.*, *Chem. Phys. Lip.* 40:89 (1986); Gregoriadis, *Liposome Technology* (1984) and Lasic, *Liposomes: from Physics to Applications* (1993)). Suitable methods include, for example, sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-
5 induced fusion of small liposome vesicles and ether-fusion methods, all of which are well known in the art.

In certain embodiments, it is desirable to target liposomes using targeting moieties that are specific to a particular cell type, tissue, and the like. Targeting of liposomes using a variety of targeting moieties (e.g., ligands, receptors, and monoclonal
10 antibodies) has been previously described (see, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044).

Examples of targeting moieties include monoclonal antibodies specific to antigens associated with neoplasms, such as prostate cancer specific antigen and MAGE. Tumors can also be diagnosed by detecting gene products resulting from the activation or
15 over-expression of oncogenes, such as ras or c-erbB2. In addition, many tumors express antigens normally expressed by fetal tissue, such as the alphafetoprotein (AFP) and carcinoembryonic antigen (CEA). Sites of viral infection can be diagnosed using various viral antigens such as hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and
20 papilloma virus antigens. Inflammation can be detected using molecules specifically recognized by surface molecules which are expressed at sites of inflammation such as integrins (e.g., VCAM-1), selectin receptors (e.g., ELAM-1) and the like.

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes lipid components, e.g.,
25 phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid derivatized bleomycin. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (see Renneisen *et al.*, *J. Biol. Chem.*, 265:16337-16342 (1990) and Leonetti *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87:2448-2451 (1990).

Assays for determining regulation of gene expression

A variety of assays can be used to determine association of a candidate gene with a selected phenotype. The activity of a particular gene regulated by a zinc finger protein can be assessed using a variety of *in vitro* and *in vivo* assays, by measuring, 5 e.g., protein or mRNA levels, product levels, enzyme activity, tumor growth; transcriptional activation or repression of a reporter gene; second messenger levels (e.g., cGMP, cAMP, IP₃, DAG, Ca²⁺); cytokine and hormone production levels; and neovascularization, using, e.g., immunoassays (e.g., ELISA and immunohistochemical assays with antibodies), hybridization assays (e.g., RNase protection, northerns, *in situ* 10 hybridization, oligonucleotide array studies), colorimetric assays, amplification assays, enzyme activity assays, tumor growth assays, phenotypic assays, cDNA arrays studies, and the like.

Zinc finger proteins are often first tested for activity *in vitro* using cultured cells, e.g., 293 cells, CHO cells, VERO cells, BHK cells, HeLa cells, COS cells, and the 15 like. Preferably, human or mouse cells are used. The zinc finger protein is often first tested using a transient expression system with a reporter gene, and then regulation of the target candidate gene is tested in cells and in animals, both *in vivo* and *ex vivo*. The zinc finger protein can be recombinantly expressed in a cell, recombinantly expressed in cells transplanted into an animal, or recombinantly expressed in a transgenic animal, as well as 20 administered as a protein to an animal or cell using delivery vehicles described below. The cells can be immobilized, be in solution, be injected into an animal, or be naturally occurring in a transgenic or non-transgenic animal.

Modulation of gene expression and association of the candidate gene with a selected phenotype is tested using one of the *in vitro* or *in vivo* assays described herein. 25 Cells or subject animals comprising the candidate genes are contacted with zinc finger proteins and compared to control genes or second candidate genes to examine the extent of phenotype modulation. For regulation of gene expression, the zinc finger protein optionally has a K_d of 200 nM or less, more preferably 100 nM or less, more preferably 50 nM, most preferably 25 nM or less.

30 The effects of the zinc finger proteins can be measured by examining any of the parameters described above. Any suitable gene expression, phenotypic, or

100-280-02660

physiological change can be used to assess the influence of a zinc finger protein. When the functional consequences are determined using intact cells or animals, one can also measure a variety of effects such as tumor growth, neovascularization, hormone release, transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots or oligonucleotide array studies), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as cGMP.

5 Examples of assays for a selected phenotype include e.g., transformation assays, e.g., changes in proliferation, anchorage dependence, growth factor dependence, foci formation, growth in soft agar, tumor proliferation in nude mice, and tumor
10 vascularization in nude mice; apoptosis assays, e.g., DNA laddering and cell death, expression of genes involved in apoptosis; signal transduction assays, e.g., changes in intracellular calcium, cAMP, cGMP, IP3, changes in hormone and neurotransmitter release; receptor assays, e.g., estrogen receptor and cell growth; growth factor assays, e.g., EPO, hypoxia and erythrocyte colony forming units assays; enzyme product assays,
15 e.g., FAD-2 induced oil desaturation; transcription assays, e.g., reporter gene assays; and protein production assays, e.g., VEGF ELISAs.

20 In one embodiment, the assay for the selected phenotype is performed *in vitro*. In one preferred *in vitro* assay format, zinc finger protein regulation of gene expression in cultured cells is examined by determining protein production using an
ELISA assay.

25 In another embodiment, zinc finger protein regulation of candidate gene expression is determined *in vitro* by measuring the level of target gene mRNA expression. The level of gene expression is measured using amplification, e.g., using PCR, LCR, or hybridization assays, e.g., northern hybridization, RNase protection, dot blotting. RNase protection is used in one embodiment. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

30 Alternatively, a reporter gene system can be devised using a target gene promoter operably linked to a reporter gene such as luciferase, green fluorescent protein, CAT, or β -gal. The reporter construct is typically co-transfected into a cultured cell.

PCT/US2003/016610

After treatment with the zinc finger protein of choice, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

Another example of an assay format useful for monitoring zinc finger protein regulation of candidate gene expression is performed *in vivo*. This assay is particularly useful for examining zinc finger proteins that inhibit expression of tumor promoting genes, genes involved in tumor support, such as neovascularization (e.g., VEGF), or that activate tumor suppressor genes such as p53. In this assay, cultured tumor cells expressing the zinc finger protein of choice are injected subcutaneously into an immune compromised mouse such as an athymic mouse, an irradiated mouse, or a SCID mouse. After a suitable length of time, preferably 4-8 weeks, tumor growth is measured, e.g., by volume or by its two largest dimensions, and compared to the control. Tumors that have statistically significant reduction (using, e.g., Student's T test) are said to have inhibited growth. Alternatively, the extent of tumor neovascularization can also be measured. Immunoassays using endothelial cell specific antibodies are used to stain for vascularization of the tumor and the number of vessels in the tumor. Tumors that have a statistically significant reduction in the number of vessels (using, e.g., Student's T test) are said to have inhibited neovascularization.

Transgenic and non-transgenic animals are also used as an embodiment for examining regulation of candidate gene expression *in vivo*. Transgenic animals typically express the zinc finger protein of choice. Alternatively, animals that transiently express the zinc finger protein of choice, or to which the zinc finger protein has been administered in a delivery vehicle, can be used. Regulation of candidate gene expression is tested using any one of the assays described herein. Animals can be observed and assayed for functional changes, e.g., challenged with drugs, mitogens, viruses, pathogens, toxins, and the like.

Transgenic mice and in vitro high throughput assays for drug discovery

A further application of the zinc finger protein technology is manipulating gene expression in cell lines and transgenic animals. Once a selected candidate gene has been associated with a phenotype, and the candidate gene has been validated as a drug

therapy target, cell and transgenic-animal based assays are developed for the purposes of high throughput drug screening. A cell line or animal expressing the candidate gene is provided with a zinc finger protein that regulates expression of the candidate gene. The zinc finger protein typically is provided as a nucleic acid encoding the zinc finger protein, 5 although it can also be administered as a protein. The cell line or animal is then contacted with test compounds to determine the effect of the compound upon the candidate gene and the selected phenotype. The zinc finger protein technology is an improvement for high throughput cell-based and animal assays, for example, because expression of the zinc finger protein can be made conditional using small molecule systems.

10 In one embodiment of a high throughput assay for therapeutics, zinc finger proteins can be used for regulation of candidate genes in cell lines or animals using the small molecule regulated systems described herein. Expression and/or function of a zinc finger-based repressor can be switched off during development and switched on at will in the cells or animals. This approach relies on the addition of the zinc finger protein
15 expressing module only; homologous recombination is not required. Because the zinc finger protein repressors are *trans* dominant, there is no concern about germline transmission or homozygosity. These issues dramatically affect the time and labor required to go from a poorly characterized gene candidate (a cDNA or EST clone) to a mouse model. This ability can be used to rapidly identify and/or validate gene targets for
20 therapeutic intervention, generate novel model systems and permit the analysis of complex physiological phenomena (development, hematopoiesis, transformation, neural function etc.). Chimeric targeted mice can be derived according to Hogan *et al.*,
Manipulating the Mouse Embryo: A Laboratory Manual, (1988); *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, ed., (1987); and Capecchi *et*
25 *al.*, *Science* 244:1288 (1989).

Gene Identification

30 The methods and compositions described herein can be used to confirm or rebut putative gene identification based on various analyses of genomic sequence. One type of analysis used for putative gene assignment is alignment of EST and/or mRNA sequences. *See*, for example, Mott *et al.* (1997) *Comput. Appl. Biosci.* 13:477-478;

100-0007-4560

Florea *et al.* (1998) *Genome Res.* **8**:967-974; Bailey *et al.* (1998) *Genome Res.* **8**:362-376. Another method for gene prediction is based on sequence homology to known genes and/or proteins. *See, for example, Birney *et al.* (1996) *Nucleic Acids Res.* **24**:2730-2739; Gelfand *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93**:9061-9066.* In addition, a number of 5 *ab initio* gene prediction algorithms are available and are known to those of skill in the art; these include but are not limited to Genescan, Genie and FGENES. *See, for example, Burge *et al.* (1997) *J. Mol. Biol.* **268**:78-94; Kulp *et al.* (1996) *ISMB* **4**:134-142; Reese *et al.* (2000) *Genome Res.* **10**:529-538; Solovyev *et al.* (1997) *ISMB* **5**:294-302.*

10 Additional gene prediction algorithms include, but are not limited to, GenScan, Grail, GrailEXP, Veil, AAT, MZEF, PROCRUSTES, PGF, GeneParser, Glimmer, HMMgene, GeneMark-HMM, Selfid, the Webgene suite, GeneMark, EuGene, Morgan, GenomeScan, Diogenes, Genlang, FGENE, FGENESH, FGENESH+, GeneID, GENMARK, Xpound, Otto, GeneFinder, GeneWise, GENEBUILDER, GLIMMER and Ensembl. These algorithms can be accessed, for example, on the Internet, as will be 15 known to those of skill in the art. *See also Haussler *et al.* (1998) *Trends Biochem. Sci.* **23**(suppl):12 and Claverie (1997) *Human Mol. Genet.* **6**:1735.*

20 Despite the existence of a large number of gene prediction algorithms (as well as additional methods of gene prediction, *see supra*), the current success rate for exon prediction in the human genome is only 70%, while the success rate for correctly identifying all exons of a human gene is a mere 20%. *See, for example, Dunham *et al.* (1999) *Nature* **402**:489-495; Guigo *et al.* (2000) *Genome Res.* **10**:1631-1642.* Additional 25 problems in eukaryotic genome annotation, based on analyses of the *Drosophila* and *Arabidopsis* genomes, are discussed by Lewis *et al.* (2000) *Curr. Opin. Struct. Biol.* **10**:349 and Pavly *et al.* (1999) *Bioinformatics* **15**:887.

30 Thus, the methods described above generate one or more putative gene sequences, whose identification as a gene must be confirmed. One method of confirmation is to test for functionality, *i.e.*, if a putative gene sequence is actually a gene, it should be possible to modulate its expression, and such modulation should be accompanied by a phenotype.

00954750-082801

Accordingly, the methods and compositions disclosed herein are used to test a putative gene prediction (*i.e.*, to identify a gene) by contacting a cell, comprising the putative gene sequence, with an exogenous molecule that, if the putative gene sequence actually encodes a gene, will bind to, and modulate expression of, the gene.

5 The cells are then assayed for at least one selected phenotype. If one or more of the selected phenotypes are observed, the putative gene sequence is identified as a gene. Thus, detection of a phenotype is indicative of a correct gene prediction.

Thus, a putative gene sequences can be used as a source of target sites for the design of one or more exogenous regulatory molecules. In a preferred embodiment, 10 the exogenous regulatory molecule is a zinc finger protein. A zinc finger protein can be designed or selected to bind, in a sequence-specific fashion, to a predetermined target site, as known in the art. For example, in one embodiment, target sites are selected and zinc finger proteins are designed to recognize such target sites, as disclosed in co-owned PCT WO 00/42219. In another embodiment, zinc finger DNA binding domains are designed 15 according to design rules disclosed in PCT WO 98/53058, WO 98/53059 and WO 98/53060. In a further embodiment, zinc finger DNA binding domains are selected as disclosed in PCT WO 98/53057 or WO 00/27878. The target site(s) can reside in any portion of the putative gene, including but not limited to putative coding regions, putative transcribed regions, overlapping the putative transcriptional startsite and within putative 20 regulatory regions.

The zinc finger protein can optionally comprise one or more functional domains, for example, as described *supra* in the section entitled “Functional domains.” Fusion proteins comprising a zinc finger DNA-binding domain and one or more functional domains (and nucleic acids encoding them) are constructed by methods known 25 in the art and described *supra*. See also co-owned PCT WO 00/41566 and WO 00/42219.

In one embodiment, a zinc finger DNA-binding domain is fused to a transcriptional activation domain. Preferred activation domains include VP16 and the p65 subunit of NF-κB. In another embodiment, a zinc finger DNA-binding domain is fused to a transcriptional repression domain. Preferred repression domains include 30 KRAB and v-erbA.

TOP SECRET - DRAFT - 650

In a further embodiment, a zinc finger DNA-binding domain is fused to a bifunctional domain (BFD). A bifunctional domain is a transcriptional regulatory domain whose activity depends upon interaction of the BFD with a second molecule. The second molecule can be any type of molecule capable of influencing the functional properties of 5 the BFD including, but not limited to, a compound, a small molecule, a peptide, a protein, a polysaccharide or a nucleic acid. An exemplary BFD is the ligand binding domain of the estrogen receptor (ER). In the presence of estradiol, the ER ligand binding domain acts as a transcriptional activator; while, in the absence of estradiol and the presence of tamoxifen or 4-hydroxy-tamoxifen, it acts as a transcriptional repressor. Another 10 example of a BFD is the thyroid hormone receptor (TR) ligand binding domain which, in the absence of ligand, acts as a transcriptional repressor and in the presence of its ligand 3,5,3'-triodo-L-thyronine (T3), acts as a transcriptional activator. An additional BFD is the glucocorticoid receptor (GR) ligand binding domain. In the presence of dexamethasone, this domain acts as a transcriptional activator; while, in the presence of 15 RU486, it acts as a transcriptional repressor. An additional exemplary BFD is the ligand binding domain of the retinoic acid receptor. In the presence of its ligand *all-trans*-retinoic acid, the retinoic acid receptor recruits a number of co-activator complexes and activates transcription. In the absence of ligand, the retinoic acid receptor is not capable of recruiting transcriptional co-activators. Additional BFDs are known to those of skill in 20 the art. *See*, for example, US Patent Nos. 5,834,266 and 5,994,313 and PCT WO 99/10508.

Following contact of a cell comprising a putative gene sequence with an exogenous molecule capable of modulating expression of the sequence if it is indeed a gene, the cell is assayed for one or more selected phenotypes, with an optional incubation 25 period intervening between contact and assay. During the incubation period, if it occurs, the cell can also be optionally subjected to one or more stimuli. Any phenotype can be used as the basis for assay; exemplary assays and phenotypes have been described *supra* in the section entitled "Introduction" and in the definition of "selected phenotype." In addition, a phenotype can comprise a change in cell growth (*e.g.*, more rapid growth or 30 slower growth), cell cycle control (*e.g.*, loss of cell cycle control, cell cycle arrest), cellular physiology (*i.e.*, energy state, membrane potential, ion flux, production of

0007-0007-0007-0007

metabolites, macromolecules, and other cellular products) or cellular response to a pathogen such as, for example, a virus, bacterium or unicellular eukaryote. Cellular responses to a pathogen can include, for example, any of the phenotypes already described. Furthermore, the same techniques can be applied to confirm the assignment of 5 a viral gene; *i.e.*, if the putative gene sequence is part of a viral genome and a cell is infected with a virus comprising the putative gene sequence.

In addition, a selected phenotype can be a change in the rate or level of expression of a RNA molecule. For example, expression of a mRNA corresponding to a putative gene sequence following contact of a cell comprising the putative gene sequence 10 with an exogenous molecule designed to activate transcription of the putative gene sequence, provides evidence that the putative gene sequence is a gene.

On a more global level, a selected phenotype can comprise a change in expression of a plurality of RNA molecules. Accordingly, in one embodiment, a phenotype can be an alteration in the transcriptional program of a cell (*i.e.*, the 15 transcriptome). Such changes in cellular transcriptional patterns can be detected by assays known in the art, including but not limited to, microarray analysis, subtractive hybridization, differential display and serial analysis of gene expression.

Dosages

20 The dose administered to a subject or a cell should be sufficient to effect the desired phenotype. Particular dosage regimens can be useful for determining phenotypic changes in an experimental setting, *e.g.*, in functional genomics studies, and in cell or animal models. The dose is determined by the efficacy and K_d of the particular zinc finger protein employed, the nuclear volume of the target cell, and the condition of 25 the cell or patient, as well as the body weight or surface area of the cell or patient to be treated. The size of the dose also is determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular compound or vector in a particular cell or patient.

The maximum effective dosage of zinc finger protein for approximately 30 99% binding to target sites is calculated to be in the range of less than about 1.5×10^5 to 1.5×10^6 copies of the specific zinc finger protein molecule per cell. The number of zinc

CONFIDENTIAL - DRAFT

finger proteins per cell for this level of binding is calculated as follows, using the volume of a HeLa cell nucleus (approximately $1000 \mu\text{m}^3$ or 10^{-12} L ; *Cell Biology*, (Altman & Katz, eds. (1976)). As the HeLa nucleus is relatively large, this dosage number is recalculated as needed using the volume of the target cell nucleus. This calculation also 5 does not take into account competition for zinc finger protein binding by other sites. This calculation also assumes that essentially all of the zinc finger protein is localized to the nucleus. A value of $100 \times K_d$ is used to calculate approximately 99% binding of to the target site, and a value of $10 \times K_d$ is used to calculate approximately 90% binding of to the target site. For this example, $K_d = 25 \text{ nM}$

10 ZFP + target site \leftrightarrow complex
 i.e., DNA + protein \leftrightarrow DNA:protein complex
 $K_d = \frac{[\text{DNA}][\text{protein}]}{[\text{DNA:protein complex}]}$
 When 50% of ZFP is bound, $K_d = [\text{protein}]$
15 So when $[\text{protein}] = 25 \text{ nM}$ and the nucleus volume is 10^{-12} L
 $[\text{protein}] = (25 \times 10^{-9} \text{ moles/L}) (10^{-12} \text{ L/nucleus}) (6 \times 10^{23} \text{ molecules/mole})$
 = 15,000 molecules/nucleus for 50% binding
 When 99% target is bound; $100 \times K_d = [\text{protein}]$
 $100 \times K_d = [\text{protein}] = 2.5 \mu\text{M}$
20 $(2.5 \times 10^{-6} \text{ moles/L}) (10^{-12} \text{ L/nucleus}) (6 \times 10^{23} \text{ molecules/mole})$
 = about 1,500,000 molecules per nucleus for 99% binding of target site.
 The appropriate dose of an expression vector encoding a zinc finger protein can also be calculated by taking into account the average rate of zinc finger protein expression from the promoter and the average rate of zinc finger protein
25 degradation in the cell. Preferably, a weak promoter such as a wild-type or mutant HSV TK is used, as described above. The dose of zinc finger protein in micrograms is calculated by taking into account the molecular weight of the particular zinc finger protein being employed.

 In determining the effective amount of the zinc finger protein to be
30 administered, circulating plasma levels of the zinc finger protein or nucleic acid encoding the zinc finger protein, potential zinc finger protein toxicities, progression of the

PCT/US2001/016650

phenotype, and the production of anti-zinc finger protein antibodies are evaluated.

Administration can be accomplished via single or divided doses.

Pharmaceutical compositions and administration

5 Zinc finger proteins and expression vectors encoding zinc finger proteins can be administered directly to the subject or cell for modulation of gene expression. Administration of effective amounts is by any of the routes normally used for introducing zinc finger protein into ultimate contact with the tissue or cell. The zinc finger proteins are administered in any suitable manner, preferably with pharmaceutically acceptable
10 carriers. Suitable methods of administering such modulators are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

15 Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions available (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed. 1985)).

20 The zinc finger proteins, nucleic acids encoding the same, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

25 Formulations suitable for parenteral administration, such as, for example, by intravenous, intramuscular, intradermal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In
30 practice, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. The formulations of

DEPTT 08200 14-650

compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

5 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

EXAMPLES

10 The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

15 Example I: Targeting human VEGF gene with zinc finger proteins for target validation

 An important consideration in target validation is to efficiently determine and accurately evaluate the relationship between a targeted gene and resulting phenotype. This example demonstrates the use of the zinc finger protein technology to validate a gene as a target for the development of therapeutic compounds that can regulate, e.g., expression of the gene or the function of the gene product. This process is based on the following simple assumptions (Figure 1).

 If a gene X is up-regulated by a ZFP-A1, which specifically targets at the X1 site, a phenotype Q is observed.

 If the gene X is up-regulated by ZFP-A2, which specifically targets at a different site X2, the same phenotype Q should be observed.

 If the gene X is down-regulated by ZFP-B1, which targets at the X3 site (X3 can be X1 or X2), a different phenotype Z should be observed.

 If the ZFP-A1, ZFP-A2, or ZFP-B1 are used to target a gene that is not involved in the phenotype Q, no phenotype change related to this gene should be observed.

The human and mouse vascular endothelial growth factor (VEGF) genes were selected for target validation in this example. VEGF is an approximately 46 kDa glycoprotein that is an endothelial cell-specific mitogen induced by hypoxia. VEGF binds to endothelial cells via interaction with tyrosine kinase receptors Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2). Since VEGF plays a very important role in angiogenesis, targeting this gene for development of therapeutics has attracted great interest. While inhibition (down-regulation) of the VEGF gene may be used for cancer and diabetic retinopathy treatments, activation (up-regulation) of the gene may be used for ischemic heart and tissue diseases. These two desired phenotypic changes make the VEGF gene ideal for target validation using zinc finger protein technology.

Testing zinc finger proteins for biochemical affinity and specificity *in vitro*

The DNA target sites for zinc finger proteins were chosen in a region surrounding the transcription site of the targeted gene. The primary targets were chosen 15 within the region approximately 1 kb upstream of the transcription initiation site, where a majority of enhancer elements are located. Each 3-finger zinc finger protein recognizes a 9-bp DNA sequence. To increase DNA-binding specificity, two 3-finger zinc finger proteins are fused together in order to target two 9-bp DNA sequences that are in a close proximity (Liu *et al. Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530 (1997)).

20 Human SP-1 or murine Zif268 transcription factors were used as a progenitor molecular for the construction of designed zinc finger proteins. The amino acid sequences (fingers), which recognize the target DNA sequence, were designed based on the “recognition rules” described herein. The designed zinc finger protein genes were constructed using a PCR-based procedure that utilizes six overlapping oligonucleotides.

25 The methods of designing and assembling zinc finger protein genes that target VEGF are detailed in co-owned PCT WO 00/41566.

The designed zinc finger protein genes were initially cloned into the pMAL-KNB vector after digesting with KpnI and BamHI (Figure 2). The pMAL-KNB vector is modified from the pMAL-c2 vector (New England Biolabs, MA). The zinc finger protein proteins were purified from bacteria and were subjected to biochemical

affinity and specificity assays. The methods for these *in vitro* assays are described herein and in co-owned PCT WO 00/41566.

Activation or repression of a luciferase promoter in transiently transfected cells

5 The zinc finger proteins with high biochemical affinity and specificity were subcloned into the KpnI and BamHI sites in pcDNA-NVF or pcDNA-NKF (Figure 2). The pcDNA-NVF construct contains a CMV promoter-controlled sequence encoding a nuclear localization signal, a herpes simplex virus VP16 activation domain, and a Flag peptide. This construct was designed to up-regulate the targeted gene when introduced
10 into mammalian cells. The pcDNA-NKF construct contains the Kruppel-associated box (KRAB) repression domain instead of VP16 domain and was used for down-regulation of the targeted genes. These constructs are described in detail in co-owned PCT WO 00/41566.

15 The reporter plasmid system is based on the pGL3-promoter and pGL3-control vectors (Promega, WI). Three tandem repeats of the zinc finger protein target sites were inserted upstream of the SV40 promoter (Figure 3). The pGLP reporters were used to evaluate the activities of the engineered zinc finger proteins for up-regulation of gene expression and the pGLC reporters were used to measure the effects of ZFP-KRAB activities inhibition of gene expression. These constructs are described in detail in co-owned PCT WO 00/41566.

20 The control plasmids used in this example are shown in Figure 2. pcDNA-NVF (or pcDNA-NKF) is a ZFP-less effector. pcV-RAN (or pcK-RAN) expresses all components except that the engineered zinc finger protein has no known DNA binding capability (Figure 2). The zinc finger protein sequence in the pcV-RAN (or pcK-RAN) constructs is:

VPGKKQHICHIQCGKVYGGHDTVVGHLRWHTGERPFMCTWSYCGKRFTAA
DEVGLHKRTHTGEKKFACPECPKRFMLVVATQLHIKTHQNKKGGS (SEQ ID

25 NO: 13), where the fingers are underlined. These control constructs were used to check the effects of the regulation domains (VP16 or KRAB), in the absence of the DNA binding domain. The pc-ZFP-cat plasmid expresses a specifically designed zinc finger protein, however the functional domain (VP16 or KRAB) was replaced with a 234 bp

100-99-88-77-66-55-44-33-22-11

fragment isolated from the chloramphenicol acetyltransferase (CAT) gene in the pcDNA3.1/CAT vector (nt1442 to 1677) (Invitrogen, CA) (Figure 2). This control plasmid was used to test whether the DNA binding domain alone has any effects on gene expression. The other controls include effectors expressing zinc finger proteins that recognize different DNA sequences and reporters containing non-specific zinc finger protein target sequences.

The following example demonstrates the effect of a designed zinc finger protein, which activates the luciferase reporter gene in 293 cells. The targeted sequence, GGGGTTGAG, is named M6-1892S and is in the promoter region of the human VEGF gene. The zinc finger protein recognizing this 9-bp DNA sequence was designed and assembled as described herein and in co-owned PCT WO 00/41566. The DNA sequence (SEQ ID NO: 14) and the amino acid sequence (SEQ ID NO: 15) of the zinc finger protein are shown below.

35 The KpnI-BamHI DNA fragment of the assembled zinc finger protein was cloned into KpnI-BamHI sites of the pMAL-KNB vector. The ability of the designed

zinc finger proteins to bind their target sites was verified by expressing and purifying recombinant proteins from *E. coli* and performing electrophoretic mobility shift assays (EMSA). The binding affinity (K_d) of the protein shown above was 20 nM, as determined by EMSA. This KpnI-BamHI ZFP fragment was then subcloned into KpnI-
5 BamHI sites of the pcDNA-NVF vector and was named pcV-VF471A. The luciferase reporter plasmid containing three tandem repeats of the M6-1892S sites was made and named pGLP-VF471x3.

All plasmid DNA was prepared using Qiagen plasmid purification kits. The human embryonic kidney 293 cells were seeded into each well of a 6-well plate with
10 a density to reach approximately 70% confluence the next day. Cells were co-transfected with 50 ng effector DNA (ZFP-expression plasmid), 900 ng reporter DNA and 100 ng pCMV-LacZ DNA using either Lipofectamine (GIBCO-BRL, MD) or GenePORTER (Gene Therapy Systems Inc, CA) transfection reagent. The co-expressed β -galactosidase activity was used a control to normalize the luciferase activity. Cell lysates were
15 harvested 40 to 48 hours after transfection. Luciferase assays were performed using the Dual-Light Luciferase and β -galactosidase Reporter Assay System (Tropix, MA). A typical luciferase assay result is shown in Figure 4.

This example demonstrated that this designed ZFP-expressing plasmid, pcV-VF471A, was able to stimulate the luciferase gene expression by 8 fold when
20 compared with control plasmid pcV-RAN, which does not possess known DNA binding capability. When the VP16 domain was replaced with a peptide, which has no transcription regulation activity, this zinc finger protein (pcV-VF471A-cat) lost its activity of trans-activating the luciferase gene. The designed zinc finger protein (pcV-VF471A) failed to activate the luciferase expression from the reporter containing a
25 different zinc finger protein binding site, indicating that the trans-activation effect is sequence specific. Therefore, the DNA binding domain (VF471A ZFP) combined with the regulation domain (VP16) in this example were able to turn on the gene at an appropriate target sites.

092057-082801

Testing a reporter containing native promoter of the targeted gene in transiently transfected cells

The difference between the simple reporter system and the native reporter system is that the native reporter plasmid construct contains the promoter of the targeted gene. A unique advantage for the native reporter system is that a single native reporter plasmid construct can be used to analyze the effects of multiple zinc finger proteins in the context of the promoter.

The pGLP-native reporter was constructed by replacing the SV40 promoter in pGL3-promoter with a DNA fragment containing the promoter and flanking sequences of the targeted gene (Figure 3). In this example, the native reporter construct of the human VEGF gene was generated by PCR-amplifying a 3319-bp fragment from the human genomic DNA. This fragment contains the VEGF promoter and its flanking regions. The VEGF ATG codon was fused to the luciferase coding region. Nest-PCR is performed for the amplification. The external primers were hVEGFU1 (5'-GAATTCTGTGCCCTCACTCCCCTGG (SEQ ID NO: 16); nt 1 to 25 based on GenBank sequence M63971) and VEGFD2 (5'-ACCGCTTACCTTGGCATGGTGGAGG (SEQ ID NO: 17); nt 3475 to 3451). The internal primer pair are hVEHFU2 (5'-ACACACCTTGCTGGTACCACCATG (SEQ ID NO: 18); nt 71 to 95, KpnI site underlined)) and VEGFD1 (5'-GCAGAAAGTcCATGGTTCGGAGGCC (SEQ ID NO: 19); nt 3413 to 3388, a T to C substitution is made to generate the underlined NcoI site). The nested PCR product was digested with KpnI and NcoI and ligated with the KpnI-NcoI vector fragment of the pGL3-promoter plasmid (Figure 3). The human VEGF native reporter plasmid was named pGLPVFH.

A similar strategy was used to amplify a 2070-bp fragment from the mouse genomic DNA. The external primers were mVEGFU2 (5'-TGTTTAGAAGATGAACCGTAAGCCT (SEQ ID NO: 20); nt 1 to 25 based on GenBank sequence U41383) and VEGFD2 (5'-ACCGCTTACCTTGGCATGGTGGAGG (SEQ ID NO: 21); nt 3475 to 3451 based on M63971). The internal primers were mVEGF

100-0920-0220-0000

(5'-GCCCCCATGGtACCCTGGCTTCAGTCCCTGGCAACA (SEQ ID NO: 22); nt 155 to 192; a C to T replacement is made to generate the underlined KpnI site) and VEGFD

(5'-GCAGAAAGTcCATGGTTCGGAGGCC (SEQ ID NO: 23); nt 3413 to 3388 based 5 on M63971; a T to C substitution is made to generate the underlined NcoI site). VEGFD2 and VEGFD1 primers were used to amplify both human and mouse genomic DNA since the sequences are highly homologous at that region (Shima *et al. J. Biol. Chem.* 271:3877 (1996)). The murine VEGF native reporter plasmid was called pGLPmVF.

10 The following example demonstrates that two designed zinc finger proteins were able to up-regulate the human VEGF native promoter gene in 293 cells. One zinc finger protein (pcV-M6-2009A) was designed to target a proximal site GAAGGGGGC located at 362-bp upstream of the transcription start site and the other one (pcV-M6-111S) was designed to target a distal site ATGGGGGTG located at 2240-nt 15 upstream of the transcription start site. Similar to the luciferase reporter assay described above, 50 to 100 ng of effector DNA are co-transfected with 900 ng of native reporter DNA and 100 ng of pCMVlacZ DNA. Luciferase activities were measured approximately 40 hours post-transfection and were shown as fold activation in Figure 5.

20 **Primary zinc finger proteins to activate or repress the endogenous human and mouse VEGF genes in cell culture**

To test whether these engineered zinc finger proteins can activate or repress the endogenous human and mouse VEGF genes in cell culture, transient transfection experiments were conducted. The human 293 cells and mouse mammary 25 epithelial cells C127I (Shima *et al., JBC* 271:3877 (1996)) express low levels of endogenous VEGF proteins, which are used to evaluate the zinc finger protein effect on VEGF activation. The human glioblastoma U87MG cells, the mouse neuroblastoma NB41 cells (Levy *et al., Growth Factors* 2:9 (1989)) and the rat glioma GS-9L cells (Conn *et al., Proc. Natl. Acad. Sci. U.S.A.* 87:1323 (1990)) express high levels of 30 endogenous VEGF proteins, which are used for testing the repression effects of the zinc finger proteins. These cells are seeded into each well of a 6-well plate with a density to

T005676 05/2015 6/0

reach approximately 70% confluence the next day. 0.1 to 1 g effector DNA are usually used to transfect the cells using either Lipofectamine or GenePORTER transfection reagent depends on the cell types. Approximately 14 hours after transfection, cells are fed with fresh medium and cultured for another 24 hours. The mediums are then 5 harvested and endogenous VEGF levels are measured using the VEGF ELISA Assay kits (R&D Systems, MN).

The VEGF M6-111S and M6-2009S ZFPs were designed as primary zinc finger proteins to test their activities in human VEGF gene regulation. The results in 10 Table 1 indicated that both primary zinc finger proteins significantly activated the human endogenous VEGF gene expression in 293 cells.

Table 1. Activation of Human Endogenous VEGF Gene by zinc finger proteins in 293 Cells

	Effector	Target	Location*	Reporter	Fold Activation
Vector control	pcV-RAN	None	N/F	pGLPVFH	1
Primary ZFP	pcV-M6-111S	ATGGGGGTC	-2252	pGLPVFH	4.1
Primary ZFP	pcV-M6-2009S	GAAGGGGGC	-363	pGLPVFH	4.5
Secondary ZFP	pcV-M6-120S	GGGGGTGCC	-2243	pGLPVFH	13.8
Secondary ZFP	pcV-M6-1878S	GAGTGTGTG	-536	pGLPVFH	4.2

15

*Distance between the target sites and the VEGF transcription initiation site.

N/F: Not found in the vicinity of the VEGF promoter region.

To repress the targeted gene, the designed zinc finger protein domains 20 were cloned into the pcDNA-NKF vector. After transfection of the DNA into the appropriate cells, the ZFP-KRAB fusion proteins can inhibit the endogenous gene as well as the cotransfected luciferase reporter gene. The example used here is pcK-M6-11S. As shown in Table 1, M6-111S ZFP recognizes the target sequence ATGGGGGTG. When the M6-111S ZFP fused to KRAB repression domain, an approximately 80% repression

on the cotransfected luciferase reporter gene expression and approximately 40% repression on the endogenous VEGF gene expression were achieved.

**Secondary zinc finger proteins to activate or repress the endogenous human and
5 mouse VEGF genes in cell culture**

To confirm that the physiological effects observed using the primary zinc finger proteins are due to the effects on the VEGF gene and not other side effects such as regulation of alternative gene targets, secondary zinc finger proteins that target the VEGF gene at sites different than that of the primary zinc finger protein were engineered. As 10 shown in Table 1, the two secondary zinc finger proteins also activate the endogenous VEGF gene expression in cultured cells. These results demonstrated that the zinc finger protein technology can be used to regulate gene expression and to validate a gene as a target for therapeutics.

15 Tertiary zinc finger proteins to target the genes not involved in VEGF physiology

To confirm that the physiological effects observed using the primary and secondary zinc finger proteins are due to the specific effects on the VEGF gene and not any non-specific DNA-binding or squelching effects, tertiary zinc finger proteins that target genes not involved in VEGF physiology are used as negative controls. For 20 example, a zinc finger protein designed for regulating human EPO gene expression is used as a specificity control (see Example II). EPO is also affected by hypoxia and thus is useful as a control for VEGF target validation using a hypoxia assay. VEGF inhibition specifically reverses diabetic retinopathy. This result validates VEGF as a molecular target for drug discovery and development.

25 Test the VEGF inhibition effect on a diabetic retinopathy model in rodents

Diabetic retinopathy is the most common cause of blindness amongst individuals of working age. Increased VEGF expression is a major contributor for the pathology of diabetic retinopathy. One of the strategies to treat this disease is to inhibit 30 endogenous VEGF gene expression using therapeutic compounds. As described above, zinc finger proteins provide the means to validate VEGF as a therapeutic target. Adeno-associate virus (AAV) and or retrovirus-based viral vectors are constructed as described

above. These virus vectors express the zinc finger proteins that are fused with the KRAB repression domain as described above. The viruses are generated, purified, and injected into the animals. The efficacy of the engineered zinc finger proteins is evaluated by suppression of retinal neovascularization as previously described (Admais *et al.*, *Arch.*

5 *Ophthalmol.* 114:66 (1996); Pierce *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:905 (1995); Aiello *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:10457 (1995); Smith *et al.*, *Invest. Ophthalmol. Vis. Sci.* 35:101, 1994). All necessary controls, including the viral vectors expressing the secondary and tertiary zinc finger proteins are also used.

10 **Test the VEGF activation effect on a peripheral artery disease model in rodents**

Stimulation of peripheral angiogenesis by VEGF to augment collateral artery development is a potentially novel form of therapy for patients with ischemic vascular disease. The same strategy described above is used to validate VEGF as a target using a mouse peripheral artery disease model. The AAV or retrovirus vectors, which 15 express the zinc finger proteins fused to VP16 activation domain, are constructed as described above. The efficacy of the zinc finger proteins are evaluated similar to the procedures described previously (Couffinhal *et al.*, *Am. J. Pathol.* 152:1667 (1998); Takeshita *et al.*, *Lab. Invst.* 75:487 (1996); Isner *et al.*, *Human Gene Therapy* 7:959(1996)). All necessary controls, including the viral vectors expressing the 20 secondary and tertiary zinc finger proteins are also used. VEGF overexpression triggers collateral artery growth. This result validates VEGF as a target for drug discovery and development.

Example II: Erythropoiesis Target Discovery

25 Mammalian erythropoiesis is regulated via stimulation of the erythroid progenitors by certain factor(s) that provide proliferation and differentiation signals. Hypoxia is a potent signal that induces the expression of genes controlling many physiologically relevant processes (Ratcliffe *et al.* *J. Exp. Biol.* 201:1153 (1998)). One of the processes is to “request” that certain tissues release a factor(s) for the production of 30 additional red blood cells. This phenomenon can be detected by stimulating different cell lines and/or tissues with hypoxic conditions, sampling the culture supernatants, and testing for the stimulation of erythrocyte colony forming units from murine bone marrow

cultures. Cell lines or tissues found to respond to hypoxia in this way likely express erythropoietic growth factors in a hypoxia inducible manner. The analysis of genes differentially expressed in such cells or tissues upon hypoxic treatment should lead to the identification of erythropoietic growth factor expressing genes. Zinc finger protein 5 technology can be used as analytical tools for such differential gene expression experiments and to validate the hypothetical erythropoietic growth factor genes.

A collection of cell types (including human hepatoma cell line, Hep3B) are cultured in appropriate medium and maintained in a humidified 5% CO₂-95% air incubator at 37°C. Hypoxic conditions are achieved by flushing 1% O₂-5% CO₂-94%

10 N₂ for 18 hours (Goldberg *et al.*, *Blood* 77:271 (1991)). The culture supernatants are harvested and tested in colony forming assay (Muller *et al.*, *Exp. Hematol.* 21:1353 (1993); Eaves & Eaves, *Blood* 52:1196 (1978)). The human hepatoma Hep3B cell line is found to produce an erythropoietic growth factor(s) upon hypoxic induction (Goldberg *et al.* *Proc. Natl. Acad. Sci. U.S.A.* 84:7972 (1987)) and this cell line is used for further 15 characterization.

One working hypothesis is that one (or more) of the cellular genes, which are responsible for stimulating red cell production, is activated upon hypoxia. This gene(s) may be identified by performing a differential gene expression experiment, such as Differential Display (GeneHunter, TN), PCR-Select cDNA Subtraction (Clontech, 20 CA), or microarray (Affymetrix, CA). The gene expression patterns of the RNA extracted from the Hep3B cells growing under normal and hypoxic conditions are compared.

It is very likely that multiple genes are up-regulated in the hypoxic cells. Approximately eighteen genes have been identified as up-regulated by hypoxia (Ratcliffe 25 *et al.*, *J. Exp. Biol.* 201:1153 (1998)). The erythropoietin (EPO) gene and the vascular endothelial growth factor (VEGF) gene, which have been extensively studied, are used in this example to demonstrate the application of the zinc finger protein technology to functional genomics and identification of the gene encoding the erythropoietic growth factor.

0007.20 S7-US3

Based on the DNA sequences of the candidate genes identified from the above experiments, primary zinc finger protein s are designed to target the DNA sequences located in a proximity of the promoters. The zinc finger protein construction and characterization process is the same as that described in the Example I. The zinc 5 finger proteins (a 3-finger one or a 6-finger protein) with high DNA-binding affinity and specificity are fused with either the HSV VP-16 activation domains or the KRAB repression domains to activate or block expression of the individual genes on the list.

These designed ZFP-VP16 constructs are individually transiently transfected into Hep3B cells using the GenePORTER transfection reagent (Gene Therapy 10 Systems Inc, CA) under the non-hypoxic condition. 48 hours post-transfection, the supernatants are collected and the colony forming assays are performed. The gene(s) that induces the red cell production upon zinc finger protein up-regulation is considered to be the gene(s) that encodes an erythropoietic growth factor. The results indicate that the erythropoietin (EPO) gene is responsible for the erythropoiesis regulation while all other 15 tested genes (including VEGF) are not. All necessary zinc finger protein control constructs described in Example I are also used in this example.

Another way to identify and validate the gene is to perform the similar experiments described above except that these zinc finger proteins are fused with the KRAB domains and the Hep3B cells are stimulated by hypoxia 14 hours post- 20 transfection. When the zinc finger proteins, which are designed to repress the EPO gene expression, are transfected into the Hep3B cells, no or reduced activity based on the colony forming assay is observed. All zinc finger proteins, which target genes other than the EPO gene, do not affect the red cell production under hypoxic induction.

To further validate the gene function, secondary zinc finger proteins, 25 which target at different sites of the EPO gene, are constructed. These secondary zinc finger proteins, when fused with VP16 activation domains, activate the EPO gene expression and stimulate the red cell production. Conversely, when fused with KRAB repression domains, these zinc finger proteins inhibit the EPO gene expression under hypoxic condition and fail to stimulate the red cell production.

Example III: Breast Cancer Target Gene Discovery

The growth of some breast tumors depends on the continued presence of the hormone estrogen. Estrogen is likely involved in the up-regulation of genes required for maintenance of the transformed phenotype. Cell lines derived from these tissues 5 (such as MCF-7, BT20 and T47D) retain this dependence on estrogen for growth in culture. Thus, it appears estrogen stimulates expression of essential genes in the dependent cell lines. The discovery of these estrogen-induced genes are useful molecular targets for the development of new drugs to treat breast cancer. The use of zinc finger proteins to identify estrogen-induced genes required for estrogen-dependent cell growth is 10 described herein. Furthermore, the newly discovered targets are validated using zinc finger proteins and appropriate controls.

Identifying ER-responsive genes

MCF-7 cells are grown in the absence of estrogen (estradiol) for short term 15 (1 week) and long term (28 weeks) to allow transcription of estradiol-induced genes to reach basal levels. Cells are propagated in 162 ml flasks, containing Dulbecco's Modified Eagle Medium (DMEM), lacking phenol red and supplemented with 10% charcoal-stripped Fetal Calf Serum (FCS) (Hyclone), 10 µg/ml insulin and 0.5 nM estradiol. Upon reaching 80% confluence, cells will be trypsinized and transferred to fresh 20 medium lacking estradiol. The flasks are incubated at 37°C in a humidified atmosphere of 5% CO₂.

Estrogen-responsive gene expression is stimulated by adding estradiol to the cells. The cells grown in the absence of estradiol are split into fresh medium lacking 25 estradiol. One flask will receive 10 nM estradiol (dissolved in ethanol) while the other will receive an equivalent amount of ethanol not containing estradiol. Both stimulated and unstimulated cells are harvested after 6 hrs.

RNA is isolated from the cells for identifying differentially expressed genes using a standard RNA isolation kit. Estrogen responsive genes are identified using one or a combination of the following methods; subtractive hybridization such as PCR- 30 Select from Clontech, differential display methods such as the READS technology offered by Genelogic, or Perkin-Elmer's GenScope, cDNA arrays such as GEM

TOP SECRET - DRAFT 6/10

technology from Incyte, or a high-density oligonucleotide matrix technologies offered by Affymetrix.

A number of differentially expressed (estradiol activated) genes should be identified. The cDNAs for these genes are sequenced and compiled into a list of 5 candidate genes. It is expected that many genes will be identified, including the estrogen receptor.

Initial validation of estrogen-responsive genes

Zinc finger proteins are engineered to target each of the individual 10 members of the list of candidate genes, as described above and in co-owned PCT WO 00/41566. The sequences of candidate genes are scanned for unique and easily targetable 9 bp sequences. This process will include searching databases for matches to previously sequenced genes in order to obtain additional sequences and to confirm the accuracy of the cDNA sequence generated above.

15 These designed zinc finger proteins are fused to functional domains, allowing both up regulation and knock-down of expression of the candidate genes, as described above. The functional domains to be employed are the Kruppel-associated box (KRAB) repression domain and the herpes simplex virus (HSV-1) VP16 activation domain.

20

Repression of candidate genes

For repressor studies, cells harboring the individual zinc finger proteins are assayed for failure to grow due to blocking estrogen-dependent functions. It has been established that estrogen receptor is essential for growth in MCF-7; hence these cells 25 should fail to grow when the ER gene or other estrogen dependent functions are targeted for down regulation.

Cells are cultured in the medium previously described with and without estradiol. Eukaryotic expression vectors, constructed to fuse the zinc finger proteins to the SV40 NLS and KRAB, are described above. Transfections are done using 30 Lipofectamine, a commercially available liposome preparation from GIBCO-BRL. All plasmid DNAs are prepared using Qiagen Midi DNA purification system. 10 g of the

effector plasmid is mixed with 100 ng Lipofectamine (50 μ l) in a total volume of 1600 μ l of Opti-MEM. A pCMV β -gal plasmid (Promega) will also be included in the DNA mixture as an internal control for transfection efficiency. Following a 30 minute incubation, 6.4 ml of DMEM is added and the mixture was layered on the cells. After 5 five hours, the DNA-Lipofectamine mixture is removed, and fresh culture medium containing 10% charcoal-stripped FCS, 10 μ g/ml insulin and 10 nM estradiol are layered on the cells.

Viability is assayed by trypan blue exclusion and monitoring growth. Cells are trypsinized, concentrated by centrifugation and resuspended at approximately 10 10^6 cells/ml. A solution of 0.4% trypan blue is added to an equal volume of cells on a hemocytometer slide. Total and stained cells are counted under a microscope. Growth is monitored by measuring DNA synthesis. Radioactive [3 H]thymidine (0.5 μ Ci at 30 Ci/mmol; Ammersham) is added and the cells are allowed to grow for an additional 17 h. The medium is removed and cells are lysed *in situ* with 1% SDS. Cell lysates are 15 precipitated with 15% trichloroacetic acid (TCA) and collected by filtration with Whatman 3M filter discs and washed with 5% TCA then ethanol. Filters are dried and thymidine incorporation is quantitated by liquid scintillation counting.

Activation of candidate genes

20 Activation of each member of the list will also be performed to assay for estrogen-independent growth of MCF-7 cells. Eukaryotic expression vectors are constructed as described above. Transfections are done using Lipofectamine, a commercially available liposome preparation from GIBCO-BRL. All plasmid DNAs are prepared using the Qiagen Midi DNA purification system. Transfection is performed as 25 described above

Viability is assayed by trypan blue exclusion and monitoring growth. Cells are trypsinized, concentrated by centrifugation and resuspended at approximately 10 6 cells/ml. A solution of 0.4% trypan blue is added to an equal volume of cells on a hemocytometer slide. Total and stained cells are counted under a microscope. Growth is 30 monitored by measuring DNA synthesis. Radioactive [3 H]thymidine (0.5 μ Ci at 30

TOP SECRET//S7-US3

Ci/mmol; Ammersham) is added and the cells are allowed to grow for an additional 17 h. The medium is removed and cells are lysed *in situ* with 1% SDS. Cell lysates are precipitated with 15% trichloroacetic acid (TCA) and collected by filtration with Whatman 3M filter discs and washed with 5% TCA then ethanol. Filters are dried and 5 thymidine incorporation is quantitated by liquid scintillation counting.

Secondary validation

Additional testing will validate candidate genes identified during this first round of repressor and activator studies. These zinc finger proteins are designed to target 10 two distinct and separated target sites in the candidate gene. Additionally, the specificity and affinity of the zinc finger proteins are improved by fusing two three finger zinc finger protein domains to form a six finger molecule that recognizes 18 bp.

Three finger zinc finger proteins are designed, produced and assayed by EMSA as described herein. In order to locate suitable sequences, for which zinc finger 15 proteins can be easily and reliably designed, additional sequencing of the candidate genes may be required. Furthermore, additional sequences may be found in nucleotide sequence databases. Target sequences are chosen so that two 9 bp sequences are within 5 bp of each other; thus allowing linking of the zinc finger protein pairs. After identifying 20 pairs of three finger zinc finger proteins that bind with acceptable affinities and specificities, the domains are linked by PCR, amplifying the domain which constitutes fingers 4-6 of the six finger molecule. A short DNA sequence encoding a peptide sequence predicted to be unstructured and flexible is added to the N-terminus of this domain during amplification.

Each construct is transiently transfected into MCF-7 cells growing in 25 culture and is scored for failure to grow (repression) or estrogen-independent growth (activation) as described above.

Target validation using xenografts

The effects of altered target gene expression on tumor growth is assessed 30 by xenografts in nude mice. The genes encoding the zinc finger proteins are cloned into adeno-associated virus (AAV) or retrovirus-based viral vectors as described above. The

TOP SECRET - 057650

zinc finger proteins are fused to either KRAB or VP16 domains. The resulting recombinant viruses are generated, purified and used to infect MCF-7 cells. These transgenic cells are introduced subcutaneously into nude mice (Bissery *et al.*, *Semin. Oncol.* 22:3-16 (1995)). Tumors are measured twice weekly in order to estimate tumor weight (Bissery *et al.*, *Semin. Oncol.* 22:3-16 (1995); Kubota *et al.*, *J. Surg. Oncol.* 64:115-121 (1997)). The experiment is allowed to progress until tumors obtain a weight of 100-300 mg or the animals die.

5 End-point assays will include macroscopic examination of the thoracic and abdominal cavities to determine probable cause of death. Additional assays will include
10 histological analysis of tissue samples and excision of tumors for weighing.

Example IV: Fatty Acid Saturation Target Discovery in Plants

Vegetable oil quality is determined in part by the degree of saturation of the component fatty acid side chains. Excessive desaturation (beyond one or two double bonds) leads to poorer quality oils that are more prone to oxidation and rancidity. Components of the biosynthetic machinery in oil producing seeds determine the degree of desaturation. Inhibiting the expression of a gene whose product is involved in fatty acid desaturation may lead to higher quality oils. Zinc finger proteins are used as probes for differential gene expression experiment in order to identify genes that play a role in 15 setting the level of fatty acid saturation. Primary, secondary and tertiary zinc finger proteins are used to validate the newly discovered gene function. Finally, transgenic 20 plants, producing higher quality oils, are produced.

Generating candidate genes through random mutagenesis

25 Starting material is either soybean (*Glycine max*) seeds or plants. Mutagenesis is performed by either chemical treatment or random DNA insertion (Katavic *et al.*, *Plant. Physiol.* 108:399-409 (1995); Martienssen, *Proc. Natl. Acad. Sci. U.S.A.* 95:2021-2026 (1998); Hohn & Puchta, *Proc. Natl. Acad. Sci. U.S.A.* 96:8321-8323 (1999); Facciotti *et al.*, *Nature Biotech.* 17:593-597 (1999)).
30 Chemical mutagenesis of seeds is performed by soaking in 0.3% (v/v) ethylmethanesulfonate (EMS) for 16 h (Haughn & Somerville, *Mol. Gen. Genet.*

204:430-434 (1986)). M₁ seeds are propagated and allowed to self-fertilize, then M₂ seeds are randomly collected and propagated followed by another round of self-fertilization to form M₃ seeds. The fatty acid composition of the seeds and resulting plants is analyzed as described below.

5 Alternatively, random DNA insertion can be performed by transposition using a number of systems developed in plants (Martienssen, *Proc. Natl. Acad. Sci. U.S.A.* 95:2021-2026 (1998)).

Identifying potential candidate genes by fatty acid and lipid analyses

10 Fatty acid and lipid composition is determined for approximately 20-30 of the M₃ seeds according to the method of Katavic (*Plant Physiol.* 108:399-409 (1995)). Mature plant tissues are also similarly analyzed. Seeds are grouped into categories according to degree of fatty acid saturation.

15 Expression profiles are generated for seeds expressing either elevated or reduced degrees of desaturation by employing one of the methods described in Example III. (Note: FAD2-1, encoding omega-6-desaturase, is expected to be a gene underexpressed in seeds that will lower levels of polyunsaturated long chain fatty acids). Once a particular gene has been identified as participating in the altered phenotype, the cDNA is selected for sequencing.

20

Initial target validation with primary zinc finger proteins

25 Zinc finger proteins are engineered to target each of the individual members of the list of candidate genes, as described above and in co-owned PCT WO 00/42219 and PCT WO 00/41566. The sequences of candidate genes are scanned for unique and easily targetable 9 bp sequences. This process includes searching databases for matches to previously sequenced genes in order to obtain additional sequences and to confirm the accuracy of the cDNA sequence generated above.

30 These designed zinc finger proteins are fused to functional domains, allowing both up regulation and knock-down of expression of the candidate genes, as described above. The functional domains to be employed are the Kruppel-associated box

TOM2000-0007.20

(KRAB) repression domain and the herpes simplex virus (HSV-1) VP16 activation domain.

The genes encoding the ZFP-functional domain fusions are cloned into a plant expression vector such as pCAMBIA1301. This vector possesses the following 5 attributes: 1) a selectable marker such as the gene encoding hygromycin resistance; 2) left and right T-DNA borders for *Agrobacterium*-mediated transformation; 3) convenient restriction sites which will allow insertion of the zinc finger protein gene downstream of desired promoters (such as CaMV 35S, napin or phaseolin promoters); 4) a plant polyadenylation signal such as Nos; 5) a GUS reporter gene.

10 Designed zinc finger proteins are tested for activity against the desired target by assaying activation or repression of reporter genes. A single plasmid that independently expresses the zinc finger protein and the reporter is used. The target sequence is inserted in the DNA near the start site for transcription for the GUS gene. Transformation of reporter constructs into tobacco callus is carried out by standard co- 15 cultivation procedures (Grayburn *et al.*, *Biotechnol.* 10:675-678 (1992)). GUS assays are conducted using a fluorometric assay (Jefferson, *Plant Mol. Biol. Rep.* 5:387-405 (1987)).

20 Zinc finger proteins that demonstrate acceptable affinities as assessed by EMSA and *in vivo* function as assessed by reporter assays are transformed into soybean somatic embryos via particle bombardment of proliferating embryogenic cultures derived from cotyledons of immature seeds (Liu *et al.*, *Plant Cell Tiss. Org. Cult.* 46:33-42 (1996)).

25 Tissues and seeds derived from 10-20 separate transformation events for each ZFP-bearing plasmid are isolated to assess fatty acid and lipid profiles. Candidate genes which produce an altered fatty acid or lipid profile when transformed with the above zinc finger proteins are selected for secondary and tertiary designs which will generate more specific zinc finger proteins.

Secondary and tertiary zinc finger proteins to further validate target in desaturation pathway

30 Additional testing is used to validate candidate genes identified during this first round of repressor and activator studies. These zinc finger proteins are designed

to target two distinct and separated target sites in the candidate gene. Additionally, the specificity and affinity of the zinc finger proteins are improved by fusing two three finger zinc finger protein domains to form a six finger molecule that recognizes 18 bp.

Three finger zinc finger proteins are designed, produced and assayed by EMSA as described herein. In order to locate suitable sequences, for which zinc finger proteins can be easily and reliably designed, additional sequencing of the candidate genes may be required. Furthermore, additional sequences may be found in nucleotide sequence databases. Target sequences are chosen so that two 9 bp sequences are within 5 bp of each other; thus allowing linking of the zinc finger protein pairs. After identifying pairs of three finger zinc finger proteins that bind with acceptable affinities and specificities, the domains are linked by PCR, amplifying the domain which constitutes fingers 4-6 of the six finger molecule. A short DNA sequence encoding a peptide sequence predicted to be unstructured and flexible is added to the N-terminus of this domain during amplification.

15 Six finger zinc finger proteins are fused to either repression or activation domains and assayed first in tobacco callus reporter studies then in soybean plants as described herein.

Candidate genes that produce altered fatty acid or lipid profiles when targeted by the secondary zinc finger proteins described above are selected for design of 20 tertiary zinc finger proteins. A second region of the gene separate from that targeted with the secondary zinc finger proteins is chosen. Again, zinc finger proteins designed to bind 18 bp are designed and tested as described herein. These zinc finger proteins are introduced into soybean and the resulting alteration on fatty acid and lipid profiles will again be examined.

25

Although the foregoing methods and compositions have been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art, in light of the teachings herein, that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.